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IN VITRO BEHAVIOUR OF TISSUE OF ADULT MAMMALIAN CENTRAL NERVOUS SYSTEM

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

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FROM

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LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

1975

DEDICATION

"[illegible],
[illegible]"

Many a time did my wife, Gilda Imma, retort with this adage when the demands of experimental design forced me to excuse myself either for going home late or spending weekends in the laboratory. The implied acceptance of the unavoidable in the quote above, perhaps, enabled her to attain a remarkable level of tolerance. It is usual to associate one's family with any merit a thesis may have while accepting responsibility for its shortcomings. I assume this role with a sincere desire to dedicate this study to my wife for her understanding and patience in the face of silent preoccupation and to our children, Babette Araba and Olaf Kodjo, who have been unfailingly denied the paternal attention due to them.

ABSTRACT

Tissue of the central nervous system of adult rhesus monkeys has been successfully maintained *in vitro* by a culture technique that had been used for the cultivation of highly differentiated tissues. Neurons and some glial cells survived for 84 days in a chemically-defined, protein-free medium which was formulated in the course of this study. Attempts to infect motor neurons in implanted fragments of the anterior horn and cerebral cortex with poliovirus type 1 were unsuccessful.

A cell-strain was established from trypsinized adult rhesus monkey cerebral tissue. The cultures, comprising choroid epithelial cells, astrocytes and microglial cells, were maintained *in vitro* by serial subcultivations. The cells retained their normal karyotype but degenerated after about six weeks. No endogenous virus was detected.

The cultures supported the growth of a number of viruses. Echovirus type 11 and Coxsackie viruses types A7 and B3 produced cytopathogenic changes typical of the picornavirus group. Reovirus type 1 produced intracytoplasmic inclusion bodies, and giant-cells were formed in monolayers infected with vaccinia and herpes simplex virus. Vaccinia-infected cells were localised by haemadsorption. Vaccinia virus affected all cell-types indiscriminately while with the other viruses, the choroid epithelial cells succumbed to infection before the other cell types.

Serological relationship between Coxsackie viruses A7 and B3 was determined by complement-fixation test. Coxsackie A7 antigen cross-reacted with anti-Coxsackie B3 serum, but no reaction was detected between Coxsackie B3 antigen and Coxsackie A7 antiserum. A hypothesis has been postulated for the antigenic structures of the two viruses to explain for this non-reciprocal cross-reaction.

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The potential usefulness of the newly-described cell strain includes the study of neurotropic viruses including the "slow viruses" and the primary isolation of viruses from clinical materials.

CONTENTS

Reprints of published material submitted with the thesis: TSIQUAYE, K.N. and ZUCKERMAN, A.J. 1974. Maintenance of adult rhesus monkey motor neurons in tissue culture. Cytobios, 9, 207-215.

: ZUCKERMAN, A.J., TSIQUAYE, K.N. and FULTON, F. 1967. Tissue culture of human embryo liver cells and the cytotoxicity of Aflatoxin B_1. British Journal of Experimental Pathology, Vol. XLVIII, 20-27.

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1. INTRODUCTION

Observations by Ross G. Harrison (1907) on the survival of frog spinal cord tissue in explant culture initiated an intense period of research on the cultivation of nervous tissue for various studies. The earliest among these were those of Ingebrigtsen (1913) on regeneration of axis cylinders severed *in vitro* and Levaditi's attempts (1913) to maintain poliomyelitis virus in explants of monkey spinal cord and ganglia.

Much later the approach was extended to include a study of the morphology, ultrastructure and behaviour of the cells of nervous tissue to elucidate the structure of this complex tissue (Hild, 1962) and Bunge *et al.* (1963).

With improved culture techniques, myelin formation was induced in cultures of surviving neurons from both the central and peripheral nervous systems. This is exemplified by the work of Bornstein and Murray (1958) who, in repeated observations of the same myelinating cultures of newborn cat and rat cerebellum, described patterns of myelin formation, maintenance and degeneration. From their observations these workers put forward the hypothesis that neuroglia are involved in myelinization and that a considerable degree of neuroglial density around the nerve fibre is a pre-requisite for myelin sheath development.

More recent studies have yielded evidence that neurons in culture retain electrophysiological activity. Hild and Tasaki (1962) have demonstrated that many neurons in cultured cerebellar tissues obtained from cat and rat can respond to electric stimuli with characteristic action potentials and discharge impulses spontaneously. Indirect evidence indicating the presence of functional synapses in culture of new-born rat spinal cord-muscle fragments has also been reported (Curtis *et al.*, 1962). Electron microscopic evidence of synaptic junctions between neurites and somas, and between one neurite and another in cord cultures has been reported (Bunge et al., 1963). These bioelectric studies have now been extended to cultures of neonatal mouse cerebral cortex where a high degree of structural and functional organisation develops after explantation in vitro (Crain, 1963, 1964; Bornstein, 1963a, 1964). Fragments of adult human brain which had been maintained in vitro have bewn reported to exhibit spontaneous rhythmic bioelectric activity (Cunningham, 1961).

Nerve cells, in general, do not multiply after the neuroblast stage in the embryo. In order to circumvent this problem in almost all explant cultures of nervous tissue which have been used for various studies, foetal or neonatal tissue has been the material of choice. In this respect, central nervous system tissues are explanted at the developmental stage which coincides with the end of the neuroblast period and the beginning of cytodifferentiation. For example, cerebellar tissue from rat, cat and mouse is explanted soon after birth, 15-19 day foetuses of rat and mouse and about 7-8 days chick embryo provide the source of favourable tissue for explantation of spinal cord. Despite these empirical stages at which nervous tissue can be explanted Lumsden (1968) states that 2-3 years are required to surmount teething troubles encountered in the setting up of organotypic cultures of nervous tissue and "when the skills, or tricks, are acquired it is often uncertain to which essentials the success is really attributable".

Cultures of nervous tissue are of potential value for the study of neurological diseases particularly those with inherent metabolic disorders and diseases with a specific viral aetiology. The diversity of viruses invovled in the encephalomyelitides and the causation of mild diseases by some of these viruses without the frequent involvement of the central nervous system prompted this study into the behaviour of mature, fully differentiated tissue of an adult mammalian CNS in vitro.

2. THE BRAIN OF RHESUS MONKEY (Macaca mulatta)

A. EXTERNAL MORPHOLOGY

1) The Cerebral Cortex (Figs. 1, 2)

The cerebral cortex of the rhesus monkey is divided into four lobes, namely the frontal, parietal, temporal and occipital, by sulci or fissures. On the lateral surface the central sulcus or the fissure of Rolando divides the frontal from the parietal lobe. Anterior to this sulcus lie the inferior and superior precentral sulci. The principal fissure or sulcus rectus, which lies anteriorly between the curve of the inferior precentral sulcus, separates the medial frontal gyrus from the inferior frontal gyrus. The superior frontal gyrus lies anterior to the precentral gyrus and medial to the superior precentral sulcus.

The postcentral gyrus, which is the most anterior gyrus of the parietal lobe, lies posterior to the central fissure. The parietal lobe is bounded anteriorly by the central fissure, posteriorly by the parieto-occipital sulcus and dorsolaterally by the deep lateral fissure (fissure of Sylvius). Buried at the bottom of the lateral fissure is a fifth lobe known as the Insula. The superior temporal gyrus lies between the lateral fissure and the superior temporal sulcus. Below the middle temporal gyrus is the inferior temporal sulcus which borders the inferior temporal gyrus.

The occipital lobe is located behind the parieto-occiptal sulcus and the prominent sulcus lunatus. This latter sulcus starts from the midline and extends laterally to the inferior occipital sulcus. The surface of the area striata constitutes the greater part of the lateral surface of the occipital lobe. This lobe is scarcely fissurated; however, the superior occipital sulcus is 'ust discernible at the pole of the occipital lobe.

FIG. 1. BRAIN OF RHESUS MONKEY (Macaca Mulatta)

DORSAL VIEW

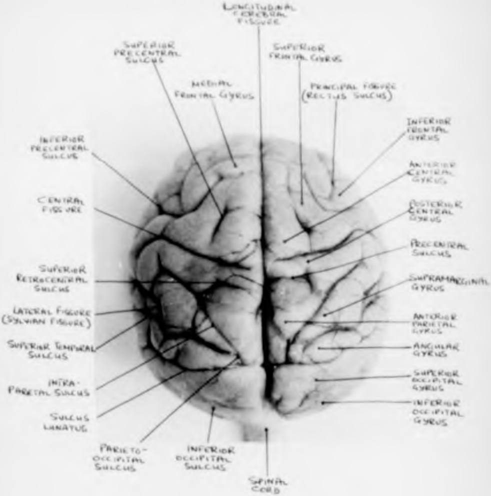
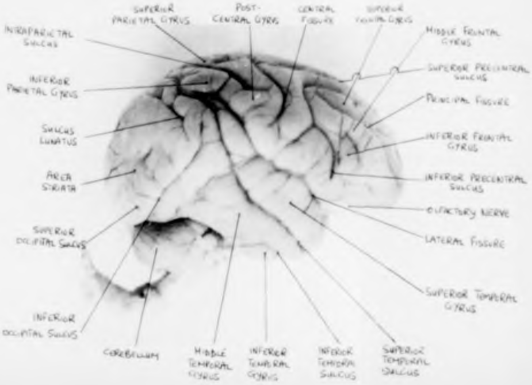


FIG. 1. BRAIN OF RHESUS MONKEY (Macaca mulatta)

LATERAL VIEW (RIGHT CEREBRAL HEMISPHERE)



The ventral surface of the brain is depicted in figure 3. In the orbital area of the frontal lobe the orbital sulcus separates the orbital gyrus from the gyrus rectus. A small shallow sulcus, the rhinal fissure, separates the uncus from the rest of the temporal lobe. Between the Sylvian fissure and the superior temporal sulcus lies the superior temporal gyrus. The posterior side of the medial temporal gyrus is marked by the medial temporal sulcus. The upper part of this gyrus joins the angular gyrus whereas the lower joins the proximal part of the occipital lobe. The inferior temporal gyrus lies between the inferior and medial temporal sulci.

The medial surface of the anterior half of each cerebral hemisphere is interrupted by the deep callosomarginal fissure (fig. 4). The superior frontal gyrus and the paracentral lobule of the motor cortex are situated dorsally to this sulcus; the callosomarginal gyrus lies on the ventral side of the sulcus. The callosal gyrus is located between the ventro-anterior part of corpus callosum and the rostral sulcus. The posterior end of the midsagittal surface reveals the anterior and posterior calcarine fissures which are ventral to the cuneus. The lingual gyrus lies dorsal to the calcarine fissure. The hyppocampal gyrus is bounded laterally and anteriorly by the rhinal fissure. The gyrus turns dorso-posteriorly to form the uncus.

ii) <u>The Cerebellum</u>

Ranson and Clark (1959) have described that the midline segment of the cerebellum is well developed in animals that are predominantly bilaterally symmetrical, for example birds in flight. On the other hand, in those animals that have evolved individual movements of the limb, the lateral portions of the cerebellum are most predominant. The cerebellum of the rhesus monkey is made up of a small, unpaired medial portion, the vermis, which is sandwiched between two well developed lateral cerebellar hemispheres.

FIG. 3. BRAIN OF RHESUS MONKEY (Macaca Mulatta)

VENTRAL VIEW

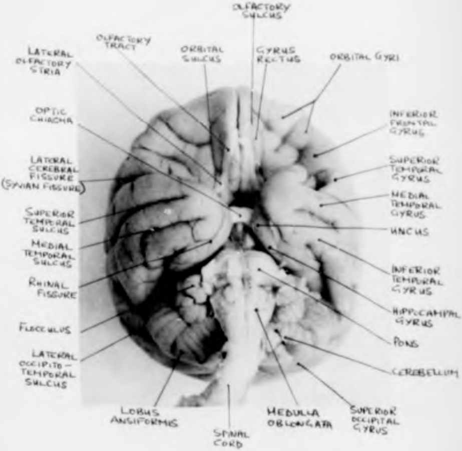
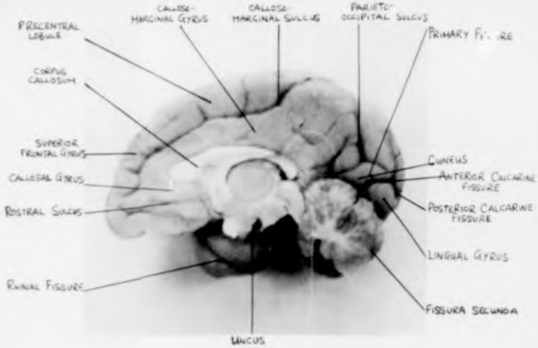


FIG. 4. BRAIN OF RHESUS MONKEY (Macaca mulatta)



MIDSAGITTAL SECTION

In the midsagittal section (Fig. 4), the vermis is seen divided into three lobes by a deep anterior fissure called the primary fissure and a deep posterior fissure, the fissura secunda. The anterior lobe is subdivided into the culmen, the central lobule and the lingula. On the medial surface, the culmen and the central lobule are separated by the deep postcentral sulcus, and the lingula and the central lobule by the post lingual sulcus (not labelled in fig. 4).

The medial lobe is made up of the lobulus simplex, the declive, the folium, the tuber and the pyramis. The prepyramidal sulcus separates the tuber from the pyramis which is posteriorly separated from the uvula by the fissura secunda. On the ventral surface, the uvulonodular sulcus separates the uvula from the nodule. The post lunata fissure is the limiting border of the lobulus simplex. The superior and inferior semilunar lobules of the lobus ansiformis are separated by the deep intercrural fissure. These lobules and the tonsilla of the lubus ansiformis constitute the lateral hemispheres of the cerebellum.

B. CELLULAR ELEMENTS OF THE BRAIN TISSUE AND THEIR CYTOLOGICAL FEATURES

The cellular population of the brain tissue comprises neurons, and neuroglia cells which fill the interstices among neuronal elements of the central nervous system and thereby provide a supporting framework for these elements. The neuroglia cells consist of protoplasmic and fibrous neuroglia, microglia and oligodendroglia.

i) Neurons

Neurons abound in great numbers in the grey matter of the central nervous system. They may be unipolar, bipolar (cells with one axon and one dendrite) and multipolar. The latter have several dendrites but only one axon. The multipolar cells comprise, for example, the fusiform and stellate cells which are found in the cerebellum, the pyramidal cells of which there are the small, medium, large and giant or Betz cells of the motor area of the cerebral cortex, and the polyhedral cells of the anterior horn of the spinal cord. Neurons can be classified as Golgi type I, that is neurons with long axons such as the anterior horn cells and the pyramidal cells of the cerebral cortex, and Golgi type II neurons which can be found in all parts of the nervous system including the cerebellum, cerebral cortex and the spinal cord. The type II neurons possess short axons which branch repeatedly and terminate in the neighbourhood of the cell body.

Neurons can be easily identified in histological sections of adult brain by their large size, the characteristic round nucleus and basophilic cytoplasm. The nucleoplasm stains lightly, giving the nucleus a vesicular appearance. Brattgard and Hydén (1952) estimated by using chemical and X-ray microradiographic methods that the nucleus contains 20-30% lipid (dry weight). But Debuch and Stammler (1956) found that the lipid content was 5-10 and that the type of lipid varied between different types of neurons. For example, they found that the nuclei of the cerebral cortex contained

9% lecithin of the total lipids whereas this type of lipid constituted 27.6% of the lipids of the cerebellar cortex. However, no significant difference was found between the nuclei of cerebral and cerebellar cortex in the content of sphingomyelin, cholesterol, gangliosides, cerebrosides and acetyl phosphatide. A number of enzymes such as acetylcholinesterase, carbonic anhydrase, cytochrome oxidase and acid phosphatase have been found in isolated nucleus of neurons (Richter and Hullins, 1950). The demonstration of enzyme activity in the nucleoplasm by available histochemical techniques has been found wanting; therefore the presence of enzyme activity has been ascribed to a film of cytoplasm which adheres to the isolated nuclei or to the activity of the nucleolus.

Heller and Elliot (1954) have estimated from their studies on brain homogenates that each nerve cell nucleus contains 7.1pg DNA. This amount accounts for the vesicular appearance of the nucleus of stained preparations of large neurons and because the DNA is distributed in a larger volume, the nucleus of a motor neuron appears relatively poor in chromatin compared to, for example, the granule cell in the cerebellum.

Electron microscopic studies of the nucleus of spinal cord motorneurons of rhesus monkey have revealed that the relatively transparent nucleoplasm is packed with irregularly dispersed particles of the order of 100-200nm. These particles were found to stain very poorly with lead. There is also a number of irregularly distributed densely stained, smaller particles of the order of 100nm (Bodian, 1964).

The nuclei of anterior horn cells, Purkinje cells and large pyramidal cells contain prominent nucleoli which are usually in the centre of the nucleus. The nucleolus is sometimes observed in an eccentric position close to the nuclear membrane. It contains a large amount of RNA and has associated with it a cap of nucleolar heterochromatin (DNA) and the sex chromatin. It contains fat, and stains with osmium tetroxide and Sudan black B.

The association of the nucleolus with protein synthesis has been demonstrated by treating nerve cells with melanonitrite. The nucleolus increases in size in response to this stimulant of protein synthesis (Hyden and Hartelius, 1948). It has been suggested that when the nucleolus is observed at the nuclear membrane, it is discharging into the cytoplasm synthesized RNA (Tewari and Bourne, 1962a, b). Electron microscopic examination of normal motor neurons of rhesus monkey by Bodian (1964) had revealed that the nucleolus, which is 4 x 10^3nm in diameter, comprises densely stained, tightly packed coils of granular material, separated by areas of less dense nucleoplasm. The granules of the coils measure about 10nm. A large inner body, composed of a network of fine fibrils and granules with no limiting membrane, is found within the nucleolus. In histological preparations of the nervous tissue, this body is seen as a small vacuole or opaque body within the nucleolus of each cell.

In most motor neurons the nucleus is deeply indented by spurs of cytoplasm which may reach close to the nucleolus. The spurs are bounded by a double membrane in continuity with the nuclear membrane and contain a number of free ribosomal particles.

Under the light microscope the neuronal cytoplasm appears structureless. De Renyi (1931) found that the cytoplasm of the spinal ganglion cells is of a soft gelatinous nature and that of the motor neurons is softer and more plastic. Geiger (1957) showed that in living nerve cells cultured in vitro the cytoplasm exhibited slow pulsatile activity accompanied by slower movements of the cytoplasmic granules to and from the nuclear membrane and to the neurites. Of the components of cytoplasm of nerve cells, the most conspicuous is the Nissl substance which, owing to its strong basophilia, is best demonstrated with basic stains such as cresyl fast violet, toluidine blue and Einarson's gallocyanine chrome-alum. In the large motor neurons of the spinal cord, large, flaky masses of the Nissl substance are observed in the cytoplasm as well as in the proximal parts of the dendrites. Barr and Bertram (1949, 1951) demonstrated that prolonged electrical stimulation of the hypoglossal nerve greatly depleted the motor neurons of their Nissl substance. In degenerating neurons, the Nissl substance undergoes chromatolysis but reappears during the process of nerve cell restoration. The Nissl substance is a ribonucleoprotein digestible with ribonuclease. Shimizu and Kumamoto (1952) have reported that glycogen particles are located between the masses of Nissl bodies.

Electron microscopic examination of motor neurons of the spinal cord of rhesus monkey revealed that the low electron dense cytoplasm contains large numbers of mitochondria, neurofilaments, Golgi complexes and several aggregates of stacked endoplasmic reticulum with associated ribosomes. The nucleus is generally surrounded by an area devoid of chromidial masses, and rosettes of free ribosomes are found in the intercisternal spaces of the Nissl bodies. A number of "dense bodies" which stain heavily with osmium tetroxide are also found in the motoneurons.

Silver impregnation techniques reveal in the perikaryon of neurons a network of fibres and bundles of these fibres run parallel to the long axis of axons and dendrites. The fibres are aggregates of neurofibrils. In the perikaryon they ramify throughout the cytoplasmic matrix between the Nissl bodies and anastomoses may occur. Two types of neurofibrils have been observed by electron microscopy. The neurotubules, which measure 20-30nm in diameter, are present in all parts of the cell, especially in the spinal ganglion cells they may form whorls around the nucleus. The second type, the neurofilaments, are found mainly in the axons; they are about 5-10nm in diameter.

ii) Neuroglia

a) The protoplasmic and fibrous astrocytes are distinguishable from the other neuroglial cells by their cytoplasmic morphology, oval and large nuclei, and size. The protoplasmic astrocytes have short, thick, varicose processes, and the fibrous are characterised by their slender, elongated processes. The nuclei range between 6-10 x 10 nm and each contains several nucleoli. The cytoplasmic processes of the fibrous astrocytes in the white matter contain bundles of fibres but in the grey matter the protoplasmic astrocytes contain less fibrous material. The "end feet" of astrocytic processes form a layer of cytoplasm which surrounds the blood vessels in the brain and where these "end feet" are in contact with the perikaryon of neurons, the astrocytes are thought to provide nutrition to the neurons. The cytoplasm of the astrocytes does not contain basophilia. It can be stained by Cajal's gold sublimate method.

The perinuclear cytoplasm, as observed in the electron microscope, exhibits numerous mitochondria, a moderate number of ribosomes and prominent channels of endoplasmic reticulum. Irregularly shaped osmophilic bodies and glycogen granules about 20 - 45nm in diameter, are found in the vascular processes of the astrocytes. The Golgi complex is well developed. Nuclear "pores", as well as the cleft of the nuclear membrane, are prominent. The fibrous bundles, which are abundantly prominent in the fibrous astrocytes, may have a diameter of $1-2 \times 10^3$nm and are made up of closely packed filaments measuring about 6-10nm. Meller and Haupt (1967) demonstrated that astrocytes are capable of producing intracellular fibrils in tissue cultures.

b) The oligodendroglial cells which are believed to be associated with the formation of myelin in the central nervous system, are the most common of the glial cells. In the grey matter they lie close to the cell bodies of the neurons except where the neuronal surfaces are covered by synaptic knobs, and they represent the most abundant interstitial cell of the spinal grey matter. In the fibre tracts of the white matter their cytoplasm invests the myelin sheaths of axons. They are characterised by round, hardly lobulated nuclei about 3-5 x 10^3nm in diameter. Apart from their small size, they differ from the astrocytes in cytological detail. The nucleus of the oligocyte is more electron dense and it contains a nucleolus which is less dense than that of the astrocyte. Most of the nucleus is surrounded by a rim of unbranched cytoplasm densely packed with membrane-bound ribosomes and rough endoplasmic reticulum. The Golgi complex is well developed and the number of mitochondria with tubular cristae is varied. No fibres are observable in the oligocytes.

c) The microglial cells have small irregularly shaped nuclei which stain so strongly that little intranuclear detail can be seen. Electron microscopy reveals electron-dense, ribosome-rich cytoplasmic matrix. The nuclear diameter ranges from 2-3 x 10^3nm and there is only a narrow rim of perinuclear cytoplasm. The cytoplasm contains endoplasmic reticulum, Golgi complex and usually phagocytic vacuoles.

3. SEROLOGICAL METHODS

A) COMPLEMENT-FIXATION TESTS

The complement-fixation test provides indirect measurements of antigen-antibody complex formation. The tests were carried out by the plate technique described by Fulton and Dumbell (1949) and Fulton (1958).

Antisera

All the antisera used were standard neutralising antisera which were kindly provided by the Director of the Central Public Health Laboratory Services, Colindale, London. Each antiserum was inactivated (56°C for ½ hour) before use.

Antigens

The antigens used in these tests were tissue culture harvests containing extracellular and intracellular virus particles. The harvests were prepared by scraping infected cells off the surface of tissue culture flasks with a rubber policeman. The cell suspension was centrifuged at 3000 rpm for 10 minutes. The sediment was then resuspended in a small volume of the superantant and ground in a glass mortar and pestle containing sterile glass powder. The cell suspension was frozen and thawed three times to release cell-associated virus particles, pooled with the bulk of the tissue culture fluid and centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and the virus particles were sedimented in an ultracentrifuge. The pellet was suspended in phosphate buffered saline pH 7.2 and stored at -50°C.

Complement

Normal guinea-pig serum was used as a source of complement. Anaesthetised guinea-pigs were bled out from excised pulmonary artery, the blood was collected and allowed to clot before it was centrifuged at 15,000 rpm for 30 minutes at +4°C. The serum was collected into separate tubes and centrifuged for 10 minutes at 3000 rpm at +4°C. It was pooled and distributed in 1ml amounts into plastic vials and stored at -50°C. Each vial contained enough complement for a test so that a fresh batch of complement was used each time.

Diluent

The diluent for all the components of a complement fixation reaction is a Veronal-NaCl buffer (Mayer et al., 1946), containing 0.145M NaCl and 0.005M Veronal buffer at pH 7.2, with 0.00015M Ca^{2+} as calcium chloride, and 0.0005M Mg^{2+} as magnesium chloride.

Indicator System

Sheep red cells collected in Alsever's solution (Burroughs Wellcome Ltd.) were stored at +4°C. Before the cells were used for the test, they were washed in the diluent two or three times by centrifugation at 3000 rpm for 5 minutes. After the final washing, a concentrated suspension of the cells was prepared in the diluent and its approximate strength was assessed by packed cell volume in a micro-haematocrit centrifuge. The concentrated cell suspension was diluted in diluent to give a 0.2% suspension of cells. This suspension was optimally sensitised by mixing equal volumes of 1 in 400 dilution of horse anti-sheep red blood cell serum (haemolysin)(Burroughs Wellcome Ltd.) and the standardised 0.2% cell suspension together rapidly. The sensitised cells were used within 10 minutes after they had been prepared.

Method

Each test consists of a three-dimensional array of reaction mixtures in which the three variables are antigen, antiserum and complement. The different levels of each variable in each test are of equal intervals on a logarithmic scale, except that one of the levels is the zero level of antigen and antiserum.

Perspex plates were ruled into 10 x 10 one inch squares. In the primary reaction mixture, the component volumes were 25µl delivered by dropping pipette. The row variable, antiserum dilutions, was made at 0.3 log intervals. Rows 1-9 accommodated nine antiserum dilutions and row 10 served as serum control (diluent only). All the squares in each row received the same amount of the particular antiserum dilution.

The column variable is a set of complement dilutions spaced at 0.2 log intervals and each square in a particular column received the same amount of complement dilution.

The third variable is the plate constant which is a chosen level of antigen. All the squares of each plate received a fixed amount of antigen dilution. Antigen dilutions were prepared at 0.3 log intervals for successive plates. The plate constant on the first plate is diluent (serum control plate).

After the plates had been set out, they were placed on a rack and kept at +4°C overnight in a humidified sealed metal box for the primary reaction to proceed.

After overnight fixation, 50µl of the indicator system were added by dropping pitette to each primary mixture and the plates were held at 37°C for 2 hours in a sealed, humid box.

At the end of the secondary reaction, 50% end-points were read by rows and recorded. These end points occurred either at a column value or as the geometric mean between two column values.

The amount of complement fixed by the antigen-antibody complex after correction for the anti- or pro-complementary activity of antigen or antiserum can be calculated from the 50% end points (Fulton, 1958). This is represented by the equation:

$$f = z - u - v + k$$

where if we let ν be the true amount of complement required for 50% lysis, then z is an estimate of ν.

And,

u is the estimate corresponding to z at the zero level of antigen

v is the estimate corresponding to z at the zero level of antiserum

k is the estimate at zero levels of antigen and antiserum

All these estimates are in µl.

Then k is the unit of complement and f/k is the number of units of complement fixed specifically.

The number of units of complement fixed will vary as a function of the amounts of antigen and antibody present in the primary mixtures. Fulton (1958) graphically illustrated this relationship by three different contour lines.

Results obtained from three-dimensional titrations and graphical estimations of maximum antiserum and antigen titres are shown in the relevant sections.

Elston (1965) has provided a method of estimating from the results of such assays the titres of antisera or antigens and their standard errors. The following is an adaptation of his method.

In the plate technique of Fulton and Dumbell (1949) where z is an estimate of ν, only three states are recognizable namely complete lysis, no lysis or partial lysis. Let θ be the proportion of estimates z denoted by partial lysis in an experiment. Then (1 - 0) is the proportion of estimates z which are denoted by the geometric mean of two levels of complement in the same experiment.

Titres are estimated from the antiserum (or antigen) maxima line which is defined as the linear relation between the maximum value of $\log_{10} f/k$ at any level of antiserum (or antigen) and the logarithm of the concentration (μl) of antiserum (or antigen). The linear equation is determined by the method of least squares.

Let x be the logarithm of the concentration of antiserum (or antigen) and let $w = \log f/k$
then, $w = \bar{w} + \beta(x - \bar{x})$
where $\bar{w}$ and $\bar{x}$ are the mean values of w and x respectively and β is the slope of the line.

$$\text{where } \beta = \frac{\sum_{j=1}^{j=n} (x_j - \bar{x})\ (w_j - \bar{w})}{\sum_{j=1}^{j=n} (x_j - \bar{x})^2}$$

The antiserum (or antigen) titre is taken from this line to be that antiserum (or antigen) concentration at which $w = 0$. At this concentration one unit of complement is fixed. This value is obtained from the equation,

$$\beta\ (x - \bar{x}) = -\bar{w}$$

Then x_t is the logarithm of the estimated titre and antilog $x_t = X_t$ is the estimated titre.

Standard error

The standard error of the estimated titre, X_t is obtained from the f values not from $w = \log f/k$ values.

Suppose we have two points (f_1 , X_1) and (f_2 , X_2) defining the antiserum (or antigen) maxima line. Usually these will be the two extremes with $f_1 = f_2$.

It is convenient to define three quantities, S_1, S_2 and S_3 in four different ways according to the relationship between u, v and k.

1\. $u \neq k = v$: $S_1 = (z_1 t)^2 + (u_1 t)^2$
: $S_2 = (z_2 t)^2 + (u_2 t)^2$
: $S_3 = (kt)^2$

2\. $u = k \neq v$: $S_1 = (z_1 t)^2 + (v_1 t)^2$
: $S_2 = (z_2 t)^2 + (v_2 t)^2$
: $S_3 = (kt)^2$

3\. $u \neq k \neq v$: $S_1 = (z_1 t)^2 + (u_1 t)^2 + (v_1 t)^2$
: $S_2 = (z_2 t)^2 + (u_2 t)^2 + (v_2 t)^2$
: $S_3 = 0$

4\. $u = k = v$: $S_1 = (z_1 t)^2$
: $S_2 = (z_2 t)^2$
: $S_3 = 4\ (kt)^2$

where z_1, u_1, v_1 are derived from f_1; z_2, u_2 and v_2 are derived from f_2; and t is either θ or $(1 - \theta)$ depending on the type of estimate. Then the variance of the estimate, X_t, is

$$S_x^2 = \frac{0.442h^2 \left[(X_2-X_t)^2 \ S_1 + (X_1-X_t)^2 \ S_2 + (X_2-X_1)^2 \ S_3 \right]}{F^2}$$

Where $F = f_1 - f_2$

95% confidence limits for X_t are $X_t \pm 2(S_x)$

B) VIRUS TITRATION AND NEUTRALISATION TESTS

Confluent monolayers of 10 days' old primary cultures of adult rhesus monkey brain were trypsinised and the cells were suspended in growth medium to a concentration of 10^5 cells/ml. The suspension was dispensed in 1ml volumes into sterile pyrex test tubes which were then stoppered with sterile white rubber bungs. The tubes were placed on a rack inclined from the horizontal position and incubated at 36°C for 3 or 4 days before they were used for virus titration and neutralisation tests.

Harvests of infected cultures were titrated in either secondary or tertiary brain cultures to determine $TCID_{50}$ titres. Ten-fold serial dilutions of virus suspension were prepared in maintenance medium. Equal volume of medium was added to each virus dilution and incubated at 36°C for 1 hour. After the incubation period, 0.2ml of each dilution was inoculated into each of six replicate cultures. Without further incubation for adsorption, 0.8ml of maintenance medium was added to each inoculum, the tubes were placed on the rack and incubated at 36°C. The cultures were examined and scored daily for CPE. $TCID_{50}$ titres were calculated by Thompson's moving average method (1947).

In neutralisation tests, neutralising antiserum diluted 1/10 in physiological saline was inactivated at 56°C for 30 minutes. Appropriate two-fold serial dilutions of antiserum were prepared in maintenance medium and to each was added equal volume of test virus dilution containing 100 $TCID_{50}$. The mixtures were incubated at 36°C for 1 hour. The medium in thetube cultures was removed, the cultures were washed with maintenance medium and 0.2ml per culture of each test mixture was dispensed into 4 replicate cultures. Into a set of similar replicates was delivered 0.2ml amounts of the lowest serum dilution mixed with an equal volume of maintenance medium - this set served as serum controls. A concurrent titration of virus was also included to ascertain that the test dose contained 100 $TCID_{50}$. 0.8ml of medium was added to each tube; the cultures were incubated at 36°C and examined daily. The titre of the neutralising antiserum was taken as the highest serum dilution which inhibited viral cytopathic effect.

4. TISSUE CULTURE OF ADULT RHESUS MONKEY NERVOUS TISSUE

A vast literature on the cultivation of nervous tissue *in vitro* has accumulated since the inception of tissue culture (Harrison, 1907, 1910). Much of the success, including Harrison's, on the cultivation of neural tissue has been achieved with foetal or neo-natal tissue. To date, few of the studies have employed adult tissue. The first of these was reported by Murray and Stout (1947) who demonstrated migration and occasional mitosis of adult neurons in cultures of adult human sympathetic ganglia. Neurons and glial-cell elements were successfully demonstrated by Costero and Pomerat (1951) and Hogue (1953) to migrate out of explant cultures of adult human cerebral and cerebellar cortex. Geiger and Behar (1953) and Geiger (1957 , 1958) were also successful in establishing cultures of neurons and glial elements derived from the cerebral and cerebellar cortex of adult humans, monkeys and rabbits. Nerve cells were also observed to migrate in the cultures; these cultures were serially maintained by subcultivations for 2 years.

As embryonic tissues were found to be more amenable to successful maintenance and growth of neurons and glial elements, a lull in the use of adult nervous tissue was, perhaps, inadvertently established until recently when Kiernan and Petit (1971) described a technique in which neurons within explants of nervous tissue of young adult rats were maintained for 2 weeks.

Cultures of newborn and adult mammalian brain have been prepared by a number of methods. Costero and Pomerat (1951) used flying coverslips in roller tubes, Murray and Stout (1947) adapted the Maximov double-coverslip assembly, Geiger and Behar (1953) and Gieger (1958) used a combination of the Carrell flask technique and the lying-drop double-coverslip Maximov method.and Bornstein (1963) employed rat-tail collagen-coated coverslips incorporated into the Maximov assembly.

In all these methods, migration of glial elements occurred leaving the more sedentary neuronal cells in situ. The cellular elements of the brain are, however, known to be interdependent on one another. Therefore, the status quo of the cellular elements needs to be maintained in any culture technique employed for the cultivation of tissue of the central nervous system. To this end, a tissue culture technique developed by Fulton (1960) was used. In this method polythene discs were used as substrate for supporting the growth of fragments of solid organs from embryos. This technique gives rapid separation of cells without destruction of the normal morphology of the tissue. Zuckerman, Tsiquaye and Fulton (1967) successfully adapted the method to the primary cultivation of human embryonic liver cells. The fibrous and supporting cellular matrices in which neurones are embedded lend a three-dimensional network suitably applicable to the technique.

The other tissue culture method, used in this study, was the standard procedure for preparing monolayers. Here it was hoped that all the cellular elements in the brain which were insensitive to proteolytic digestion could be co-cultivated serially to determine, in addition to the use for virus studies, whether any transformation of the cells would occur with time.

A. Maintenance of Tissue Fragments of the Central Nervous System on Polythene Discs

Materials and Methods

Animals: Young adult Rhesus monkeys (Macaca mulatta) were kindly supplied by Dr. F.T. Perkins of the National Insitute for Biological Standards and Control, Medical Research Council. The monkeys were up to 6lb in weight and about two and a half years of age when they were received from Uttar Pradesh, India.

During their first week in quarantine at the National Institute the animals were given chlortetracycline 25mg/kg per monkey in the drinking water. They were tuberculin-tested and clinically examined for monkey B virus. When any animal showed signs of illness, a course of antibiotics such as chloramphenicol, streptomycin or penicillin was administered depending on the symptoms. The above details were kindly supplied by Mr. E. Hartley in a personal communication.

Maintenance Medium: Media that have been shown by most investigators to support survival and growth of neural tissue, particularly of foetal and neonatal origin, proved to be detrimental to nervous tissue obtained from adult rhesus monkeys and baboons. These media contain various combinations of foetal calf serum or horse serum and Eagle's MEM, or Simm's X7, bovine serum ultrafiltrate, human placental cord serum or human ascitic fluid and embryo extract.

After a fruitless search over a period of 15 months for a medium in which to maintain adult nervous tissue, a chemically defined, protein-free medium was formulated with the aim of mimicking the composition of cerebrospinal fluid.

The maintenance medium, which has been designated BA16-1 and has proved successful, consists of a balanced salt solution, ½ x Eagle's minimum essential amino acids and ½ x Eagle's vitamins. Details of the composition of the medium are shown in Tables I and II.

The composition of the salt solution was based on the mean ionic concentrations of electrolytes found in human cerebrospinal fluid (Documenta Geigy, 7th edition). "Analar" grade chemicals were obtained from British Drug Houses Chemicals Ltd. A single strength BSS was diluted from a concentrate (20x) which was prepared in autoclaved glass-distilled water and sterilised by membrane filtration.

Eagle's amino acids in concentrated form (50x) and vitamins (100x) were obtained from Gibco-Biocult Laboratories, Scotland.

Table 1

Composition of Balanced Salt Solution

	mg/litre
NaCl	6800
KCl	224.0
$CaCl_2.2H_2O$	167.8
$MgSO_4.7H_2O$	275.0
$NaH_2PO_4.2H_2O$	80.56
Glucose	6000

Table 1a

(Total ionic conc^{ns}, assuming complete dissociation of solutes)

Ion	meq/l
Na^+	116.86
K^+	3.00
Ca^{++}	2.28
Mg^{++}	2.23
$HPO_4^=/P$	1.03/16*
$SO_4^=/S$	2.23/3.58*
Cl^-	121.6

* Values are in mg/l

Table 2

Maintenance Medium, BA16-1

Ingredient	Volume (ml)
Balanced salt solution (20x)	5.0
Glass-distilled water	93.0
Eagle's minimum essential amino acids (50x)	1.0
Glutamine (200mM)	0.5
Eagle's vitamins (100x)	0.5
	100.00

Table 2a

BA16-2 pH 6.3-6.5

Ingredient	Volume (ml)
Balanced salt solution (20x)	5.0
Glass-distilled water	88.0
Eagle's amino acids (50x)	1.0
*Arginine (1050mg%)	3.0
*Lysine (580mg%)	2.0
Glutamine (200mM)	0.5
Eagle's vitamins (100x)	0.5

*Chemicals were obtained from Sigma, London

No antibiotics or phenol red were added to the maintenance medium, BA16-1 and medium BA16-2.

The pH of medium BA16-1 was between 2.6 and 3.0; this low value was due to the acidity of the commercial solution of amino acids, which include the hydrochloride derivatives of arginine, lysine and histidine. I have prepared and used a solution of amino acids with these three components as free base in a medium having a pH of 7.30, but without success. It was obvious then that HCl was needed in the medium.

Various buffering systems, including CO_2/HCO_3^- and zwitterionic compounds (MOPS, TRIS, HEPES, PIPES, BES) (Good *et al.*, 1966) were also found unsatisfactory when an attempt was made to increase the pH of the maintenance medium. However, addition of solutions of arginine and lysine gave a pH value of 6.3-6.5 (Table 2a). Cultures maintained in this medium and examined up to 14 days revealed that most of the neurons remained viable as judged by their morphological appearance after staining.

Tissue: The animals were injected intramuscularly with 0.3ml of sterile aqueous solution of 10mg/ml Sernylan. After they had been completely anaesthetised the monkeys were drenched in a solution of 10% Chloros; 50ml of blood were obtained by cardiac puncture and the kidneys were removed aseptically for the preparation of primary cultures.

The back of the animal was thoroughly wiped with cotton wool to dry the fur and the skin removed with sterile instruments. The vertebral column was cut open and the spinal cord with the meninges and the dorsal root ganglia intact were transferred aseptically to a sterile plastic petri dish. The lumbar and cervical enlargements, each about 6.5cm long, were placed in separate dishes. Each piece of the cord was bisected by cutting through the posterior median sulcus, the central canal and the anterior median fissure with a sterile scalpel blade. The meninges with the root ganglia still attached were separated, and each half of the cord was washed in three changes of medium BA16-1 (50ml each) in sterile plastic petri dishes. Each tissue was then put into a sterile 25ml universal container full of medium BA16-1 for transportation. The procedure for other parts of the CNS was similar. The medulla oblangata was divided longitudinally into two, the vermis of the cerebellum and slices of the cerebral cortex were obtained bu cutting sagitally with a sterile pair of scissors. Since most of the study was carried out with spinal cord tissue, detailed description of the culture technique will be devoted to this tissue.

Preparation of Cultures: The processing of the tissue began two hours after removal from the monkey. A piece of spinal cord was removed from the transport medium and placed in a sterile glass petri dish. The white matter was carefully pulled apart with a pair of sterile scalpel blades so that a strip of anterior horn grey matter with its underlying white matter was exposed. As much of the white matter as possible was dissected but no attempt was made to remove it completely. After dissection the tissue was moistened by immersing it briefly in the transport medium. Each strip of tissue was cut into about twenty pieces each measuring about 3-4mm in length.

The tissue was implanted on polythene discs. The principles and details of the technique have been described previously by Fulton (1960). A brief description of the procedure is given here. Pieces of tissue were arranged in rows of four about 1 inch apart on a square perspex plate (5" x 5") which had previously been sterilised by irradiation with UV light. Each plate accommodated 16 tissue fragments. On to each fragment was placed a polythene disc, coated on one side immediately before use, with a mixture of citrated mouse plasma and 2% $CaCl_2$ solution. The ratio of plasma to $CaCl_2$ was 3:1. When all the tissue fragments were covered with discs the plate was turned over and laid on a pad of sterile blotting paper. Gentle pressure was exerted manually on the plate to squeeze out excess fluid (plasma-$CaCl_2$ mixture) and simultaneously to flatten the tissue. After about 3-5 minutes when the plasma was clotted, the plate was turned over. 0.2ml of medium was deposited close to each disc, and with a pair of fine sterile forceps was made to run under each disc, which then, with its tissue coating, floated on the medium.

The discs were then transferred to the tissue culture plate. Each of the 16 cups was filled with 1ml of medium before a disc was placed on it. The squashed tissue had a thickness of 2-4 layers of cells.

The tissue culture plates were placed in a desiccator and incubated at 36°C. The tap on the desiccator was left open so that there was a free gaseous exchange with the atmosphere. The medium was changed weekly either by pipetting off the medium and replacing it with fresh medium or transferring the cultures into another set of cups containing fresh medium.

Staining: In general, for different histological techniques empolying the basic dyes Einarson's chrone alum gallocyanin, toluidine blue, cresyl fast violet and azure-eosinate were used to test for viability and identify the population of cells. Cresyl fast violet and azure-eosinate stains were found superior to the others and were used routinely to identify neurons by the presence of cytoplasmic basophilia.

Acetate buffered cresyl fast violet solution pH 4.5 (Manns, 1960) was used at a final concentration of 0.02% The discs were stained at 60° for 1 hour and then allowed to cool. They were washed briefly in distilled water, dehydrated through several changes of 95% alcohol until no more excess stain was removed, and absolute alcohol. Clearing was done in two changes of 25% alcohol in xylene, then xylene and mounted in DPX.

Lillie's azure-eosinate stain was used to detect necrotic changes in neurons. Discs were stained in buffered azure-eosinate (pH 4.1) at 60° for 2 hours during which complete penetration of the stain into the deeper layers of the tissue took place. After staining the discs were rinsed briefly in distilled water and dehydrated with several changes of acetone.

They were then cleared in two changes of acetone-xylene mixture (1:1) and two changes of xylene. The discs were mounted in DPX.

In all the staining methods discs were fixed in 10% formol-saline for 10 minutes and sometimes for longer periods ranging up to about 10 hours. They were then washed in four changes of distilled water for 10 minutes before staining. More often than not the tissue separated from the discs during the clearing stages. This, however, did not impair the mounting of the tissue, which was thick enough to be manipulated with a pair of forceps. The side of the tissue to which the stain had been applied was mounted uppermost on the slide. This was important for microscopic examination since there was a gradient in the depth of staining of the different layers of cells.

Observations: Since Nissl substance contains a strongly acid protein, it was argued that survival and maintenance of fully differentiated mature neurons would be ensured if tissue of the CNS was maintained *in vitro* in a fairly acid medium. This assumption was partly borne out when preliminary experiments were carried out with Eagle's minimum essential medium without sodium bicarbonate (pH4.0). Neurons retained their characteristic morphological appearance but the Nissl bodies remained granular and diffuse during the 3 weeks of observation.

Addition of small volumes of 7.5% sodium bicarbonate solution to the medium to raise the pH to 7.3 was found to be detrimental to the survival of the nerve cells. After an overnight incubation period of 15-19 hours in the CO_2/HCO_3^- buffered medium, nearly all the neurons in the explant had undergone complete chromatolysis. The nerve cells were observed as ghost-cells with grossly shrunken nuclei. Cells that had not yet reached the last stages of degeneration stained very weakly with cresyl fast violet; their nuclei were filled with darkly stained granules, the product of nucleolar disintegration. Some of these granules were often seen as specks along the nuclear membrane.

The development of medium BA16-1 was dictated by repeated failure to maintain neurons in various media which had been reported to support the growth of neurons in organotypic cultures of CNS obtained from embryos and very young animals. In media such as Eagle's MEM containing various concentrations of foetal calf serum or horse serum or Simm's X7 containing bovine serum ultrafiltrate, human placental cord serum or human ascitic fluid, and embryo extract, the chromatolytic changes were often severe. The number of healthy looking neurons in tissue fragments stained and examined immediately after implantation on to polythene discs was small and the staining reaction very weak. Their nuclei were variable in shape and eccentrically located in the perikarya. The Nissl bodies were dispersed and dust-like, and the short, rudimentary dendrites were barely visible. After overnight incubation neurons had disappeared. A few cells stained very weakly with cresyl fast violet; the cytoplasmic basophilia was finely granular and staining was much less intense than that observed in 0 hour preparations. With azure-eosinate stain cells were hardly distinguishable from the pink background.

Tissues transported and maintained in medium BA16-1, however, were morphologically characteristic of motor neurons, showing cellular variation in shape (multipolar, pyramidal or triangular and elongated) and size (large, medium and small).

Immediately after implantation the nuclei of most of the neuronal population were either oval or round with a clear vesicular area. They were centrally located in the perikarya, and each contained a round and darkly stained nucleolus within which could be seen an opaque body or small vacuole variously known, for example, as nucleololus (Dutta et al., 1961). Figure 5 shows a group of motor neurons which had been maintained in culture for 24 hours.

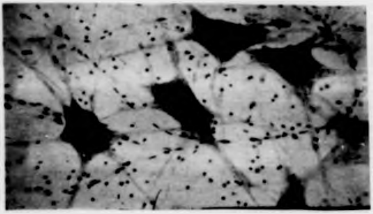


Fig. 5 . Motor neurons of the anterior horn of the spinal cord of adult rhesus monkey. 24 hours after implantation on polythene discs. Stained with cresyl fast violet.

Fig. 6 . Aging cultures of motor neurons maintained in medium BA16-1. Stained with buffered azure-eosinate stain.
Above: 63 day-old culture.
Below: 84 day-old culture.

In the cytoplasm of these cells the Nissl substance stained intensely, was flaky, discrete, and conspicuous in the dendrites, some of which measured about 270μ in length (Figs. 5 and 7). Axons were infrequently seen in these preparations; when these were thought to be present in freshly prepared cultures, they were identified by the absence of Nissl substance in the origin of the process (axon hillock).

In very large multipolar motor neurons and small cells with few or no processes, the chromidial material was granular but not diffuse. These cells were not thought to be in stage I chromatolytic state because the nuclei and nucleoli were centrally placed or very nearly so and there was no viscous zone of acidophil cytoplasm adjacent to the nuclei. Cells were also present in some of the tissue fragments with centrally located nuclei and flaky Nissl substance. But the nucleoplasm had retracted into an oval shape around a darkly stained nucleolus and was bound by a clear area, the limits of which must have been the pre-existing, delineating nuclear membrane.

Most of these cellular features were faithfully and consistently present in specimens stained and examined weekly up to 84 days of incubation. The features in aging cultures which were observed included the progressive loss of Nissl substance by dendrites, their borders became less distinct, and the microglial cells stained less intensely than younger cultures. (Fig. 6).

The effects on the nerve cells of buffering medium BA16-1 with CO_2/HCO_3 were reproducible and these have been described above. Zwitterionic compounds used as buffers produced within 24 hours loss of cytoplasmic basophilia and shrinkage of the nuclei of neurons.

So far medium BA16-2 (Table 2a) has been the only medium with a pH value higher than 3, that has maintained nerve cells alive for 14 days in these preparations.

Media BA16-1 and BA16-2 have been found to function best

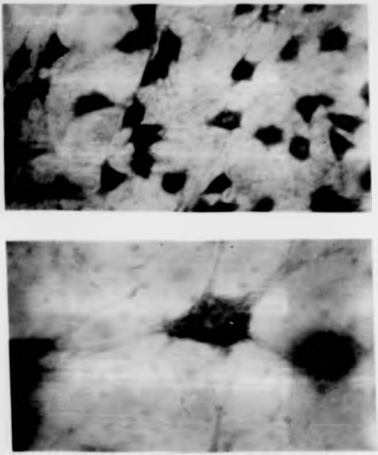


Fig. 7 . Fourteen day-old culture of rhesus monkey motor neurons in medium BA16-2. Cells had been maintained for 6 days in medium BA16-1 prior to transfer to medium BA16-2 (pH 6.4). Stained with buffered azure-eosinate stain.

Above: Low magnification.

Below: High magnification of part of figure above.

after they were prepared and stored at room temperature for a minimum period of 3 days for BA16-1 and more than a week for BA16-2 before use. Tissue transported and maintained in medium BA16-2 from the onset degenerated rapidly. However, if the tissue was initially maintained in medium BA16-1 for a week or more the survival in medium BA16-2 was greatly improved.

Susceptibility of Neurons to Virus Infection

The predisposition of poliovirus to affect motor neurons makes it a suitable model virus for testing the susceptibility of the nerve cells maintained in culture on polythene discs to virus infection. The neurovirulent strain of poliovirus type 1 (Mahoney) was obtained from Dr. McGraith of the National Institute for Biological Standards and Control, Hampstead, London.

Tissue fragments on polythene discs were transferred to medium BA16-2 (pH 6.5) for periods ranging from 24 hours to 4 days. The cultures were inoculated with virus suspension diluted to contain 2 x 10^6 $TCID_{50}$/ml of medium BA16-2. Since the distribution of neurones in the fragments varied from disc to disc, this inoculum was estimated, on the average, to be in great excess of the number of nerve cells per disc. After adsorption periods ranging from 1 hour to 24 hours, the discs were washed with three changes of media, placed in tissue culture plates containing fresh medium and incubated at 36°C for 10 days. Samples of inoculated and uninoculated discs were removed at intervals and stained by histological techniques and by the indirect fluorescent-antibody technique. Similar samples were removed and the tissue fragments were homogenized and frozen and thawed three times. The suspension was centrifuged at 3000 rpm for 10 minutes and the supernatant fluid was assayed by infectivity titration in secondary cultures of rhesus monkey kidney cells grown in test tubes.

Observations: Microscopic examination of innculated and control preparations stained with buffered azure-eosinate (pH 4.1) did not reveal necrotic changes in the neurones and no inclusion body was observed in any of the inoculated preparations.

No antigenic material was detected in inoculated tissue stained by the Sandwich technique and examined by fluorescence microscopy. Furthermore, infectivity titrations of homogenates of inoculated tissue fragments and of tissue culture fluids in rhesus monkey kidney cells failed to detect virus in the tissue fragments or released into the medium.

The stability of the virus at 36°C in the medium BA16-2 was tested. Aliquots of the inoculum in the medium were removed at intervals up to 24 hours and titrated in rhesus monkey kidney cells. The results showed that there was a fall in titre of 1.5 log at the end of 24 hours.

It was evident from these results that the surface structure of the nerve cells for virus infection had altered in some respects. For a cell to be susceptible to virus infection, it must be capable of adsorbing virus to the membrane. Adsorption is followed by transporting the virus across the membrane either by "Viropexis" (Fazekas de St. Groth, 1948) or by some unknown mechanism, uncoating the nucleic acid and then replicating virus particles. It has been suggested that the presence or absence of receptor sites for the initial adsorption of virus on to the cell membrane may be a very important factor in susceptibility.

Francis and Chu (1953) found that the neurovirulent strain of poliovirus type 2 adsorbed to primate, but not to non-primate, brain tissues. Primate cells have also been shown to adsorb poliovirus efficiently while cells of non-primate origin failed to adsorb the virus (McLaren et al., 1959). Harter and Choppin (1965) demonstrated that tissue homogenates of central nervous system derived from the cerebral cortex and spinal cord of man and rhesus monkey were capable of adsorbing neurovirulent and attenuated strains of poliovirus. It is contended that the unsuccessful attempts to initiate infection of the motor neurons with poliovirus type 1 (Mahoney) was possibly due to the destruction of specific receptors when the tissue fragments were maintained in medium BA16-1. Adsorption of many viruses appears to involve the formation of ionic bonds between complementary charges on the attachment sites of virions and on the cell receptors. Thus adsorption is inhibited by low or high pH wher one kind of the interacting group loses ionization. In addition low pH may affect the optimal physiological state of the receptors.

The viability of the cultures is considered under the section devoted to the discussion.

(B) MONOLAYER CULTURES DERIVED FROM ADULT RHESUS MONKEY BRAIN CELLS

EXPERIMENTAL

The growth medium used for the preparation of monolayer cultures was medium 8A16-1 buffered with sodium bicarbonate and supplemented with 10% inactivated foetal calf serum. The composition of the medium is given in Table 3 .

Whole brain from completely anaesthetised adult rhesus monkey was removed aseptically into a sterile plastic petri dish. A sagittal section 2 inches across the central gyrus was obtained. The tissue was washed three times with a total volume of 100ml of the growth medium. It was cut into $5mm^3$ fragments and transferred to a glass bottle containing 100ml of growth medium for transportation.

The processing of the tissue for the preparation of primary cultures began 1½ hours after removal from the monkey. The suspension of tissue fragments was transferred to a sterile Erlenmeyer flask containing a sterile bar magnet. The flask was placed on a magnetic mixer and the fragments were stirred at a slow rate for a period of 20 minutes. After removal from the mixer, the flask was allowed to stand for the tissue fragments to settle. The supernatant fluid was decanted and discarded. 100ml of 0.25% trypsin in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution was added to the fragments, stirred for 20 minutes and the supernatant again discarded. The trypsinisation procedures that followed were carried out for 10 minute periods. The cell suspension from each cycle was collected into 250ml centrifuge bottle and immediately centrifuged at +4°C at 800rpm for 10 minutes. The trypsin solution was discarded and the packed cells were suspended in 20ml of growth medium. Cell suspensions from three more cycles of trypsinisation were similarly treated. A pool of the suspensions was aspirated several times to disaggregate any lumps of cells and strained through a sterile gauze. The final cell suspension was centrifuged for 10 minutes at 800 rpm at +4°C. The loose pellet was aspirated vigorously in 150ml of growth medium. 5ml of the suspension was seeded into $25cm^2$ Falcon flasks and

Table 3

Growth medium for Monolayer Cultures

Ingredients	Volume (ml)
Balanced salt solution (x20) (see table 1)	5.0
Sterile glass-distilled water	78.8
Eagle's minimum essential medium (x50)	1.0
Glutamine (200mM)	0.5
Eagle's vitamins (x100)	0.5
Phenol red (0.5% w/v)	0.2
Sodium bicarbonate (7.5% solution)	3.0
† Penicillin & Streptomycin mixture	1.0
* Foetal calf serum	10.0
	100.0

* The maintenance medium used for virus studies contained 5% of foetal calf serum

† (10^4 units penicillin and 10^4 μg streptomycin)

incubated at 36°C. After 24 hours incubation, the flasks were removed and the loose connective tissue suspension was discarded. Each flask was gently washed with two changes of 5ml volumes of growth medium. Finally, 2ml of medium was delivered into each flask and incubated at 36°C. The medium was replaced with fresh growth medium every 2-3 days.

RESULTS

Phase-contrast microscopy of 2-3 day old cultures showed islands of choroid epithelial cells, and spindle-shaped and triangular-shaped cells. The cell had an oval or round nucleus with a single nucleolus. Another type of cell was identified as oligodendroglia by the thin rim of cytoplasm surrounding the nucleus. Most of the oligodendrocytes, however, came off the substrate with time. Sequential observation of the few which remained attached to the substrate revealed the development of cytoplasmic extensions at both ends. These extensions eventually tapered off into bi-polar, spindle-shaped cells.

The cultures grew into extensive sheets comprising a mixed population of epithelial cells, spindle-shaped (or bi-polar) and multipolar cells. Staining of 7 day – old cultures by Cajal's gold sublimate method identified the cells other than the choroid epithelial cells as glial.

By the 12th-14th day following the preparation of cultures, the cells had formed a confluent monolayer which consisted of the following morphologically different cellular elements:

a) epithelial cells with well defined nuclei and nucleoli (Figs. 8, 9).

b) glial cells consisting of

(i) oligodendroglia elements which were identifiable from the other cells by their globose form and rich granular content which produce a halo round the perinuclear cytoplasm when observed under phase-contrast microscopy (Pomerat and Costero, 1956). These cells have narrow processes originating from the perinuclear cytoplasm and (ii) multipolar cells which gave a staining reaction characteristic of neuroglial elements. In aging cultures which had never been subcultured, these two types of glial elements developed into networks in some fields (Fig. 9).

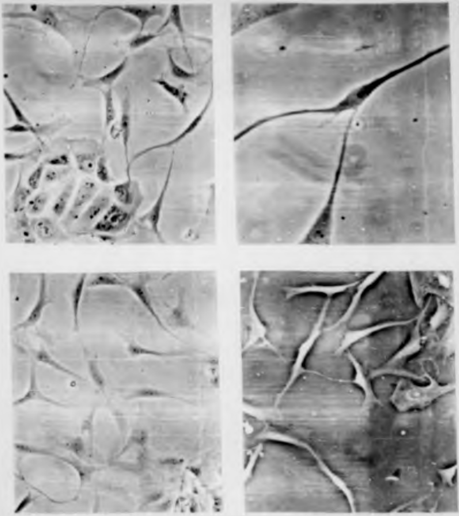


Fig. 8 . Cultures derived from adult rhesus monkey brain cells. Phase contrast microscopy.

Top left: 7 day-old culture showing a mixed cell population.
Note group of choroid epithelial cells.

Top right: Bipolar (oligodendroglia?) cell in 7 day-old cultures.

Bottom left: Multipolar cells (astrocytes?) in 10 day-old culture.

Bottom right: Similar 10 day-old culture. Negative phase contrast.

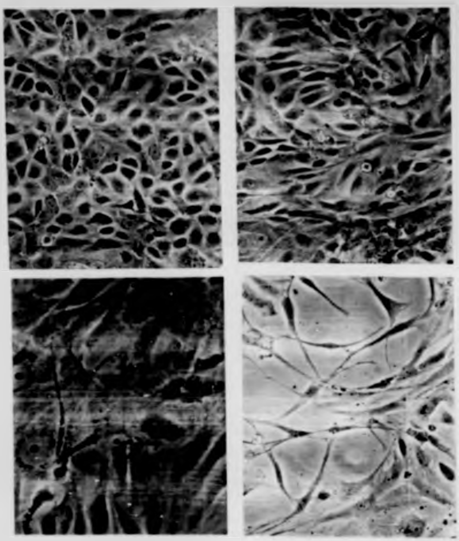


Fig. 9 . Phase contrast photomicrographs of cultures derived from adult rhesus monkey brain cells.

Top left: Areas of confluent monolayer; mainly choroid epithelial cells.

Top right: Similar culture as left; area of mixed cell type.

Bottom left: Similar cultures (high magnification). Note astrocyte with prominent nucleus and cytoplasmic granules.

Bottom right: 26 day-old culture showing network-like appearance of astrocytic and oligocytic elements.

After the growth of the primary, confluent monolayer, the cultures were routinely subcultivated at a split ratio of 1:2 every third or fourth day. The procedure involved treating the monolayer with 2ml of warmed solution of 0.25% trypsin in Ca^{2+} and Mg^{2+} free Hank's BSS for 1 minute. The solution was decanted and the flasks were incubated at 36°C. When the cells came off the substrate, they were suspended in 2ml of growth medium and divided into two flasks. After the flasks had each received 4ml of growth medium, they were incubated at 36°C. The medium was changed once before the cultures were subcultured again.

The life-span of the cultures varied from 5-6 weeks when the cells degenerated. The initial stages of the degeneration process were sign-posted by the appearance of multinucleated cells, generally at about the fifth week. Loss of the cultures began with patches of degenerating cells.

Investigative studies were carried out to determine whether the appearance of giant cells was due to replication of endogenous virus. Samples of cultures were frozen at -50°C when multinucleated cells first appeared and also when degeneration of the cultures was well established. The cells from these samples were harvested by repeated freezing and thawing, followed by centrifugation for 10 minutes at 3000 rpm. The supernatant fluids were pooled and inoculated into HeLa and Vero cells and into 1-3 day-old suckling mice No evidence of cytopathic effect in the cultures was observed. The inoculated suckling mice remained healthy till they were weaned and for three weeks afterwards when the observation was terminated. The harvests were also tested for haemagglutinins with chick, turkey, guinea-pig and human O erythrocytes. In one experiment, Tylocine, an anti-mycoplasma agent obtained from Gibco-Bio-cult Ltd., Scotland, was incorporated in the growth medium at a concentration of 60ug/ml. The medium was used for the preparation of primary cultures and for subcultivation. From about the fifth week the cultures followed the characteristic pattern of degeneration

Fig. 10 . A giant cell with 6 nuclei usually seen in 4 to 5 week old normal cultures derived from adult rhesus monkey brain cells. Acridine orange staining. x640

described above. It is suggested that failure to detect any virus in the cell-free extracts indicated that the appearance of giant cells and the degeneration which followed represented a "normal" terminal phase of these cultures derived from adult rhesus monkey brain cells.

As a cell enters the S phase of its cycle, it increases in size until by the end of the G_2 phase it is twice its original size. Since the formation of multinucleated cells did not seem to have a viral aetiology, it is conceivable that a cell entering the M phase, during which mitosis and cell division occur, might fail to complete the final stages of its cycle by not dividing into two daughter cells. The result would be the formation of a bi-nucleated cell. Such a cell could go through repeated cycles of normal growth but unable to divide. A state could therefore arise where the cell continued to increase in size with the accumulation of nuclei which, under normal cell growth, would be destined for single daughter cells. Fig.10 shows a giant cell with 6 nuclei. Gigantic cells with as many as 20 nuclei have been observed in cultures entering senescence. The degeneration of the cells by the 6th week in culture might represent a cytocidal sequelae ensuing the formation of such gigantic cells which could not cope with the package of genetic material accommodated in them.

Growth of Cultures Derived from Adult Rhesus Monkey Brain Cells at 36°C

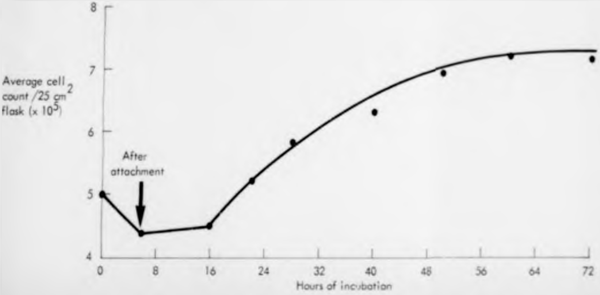
Eighteen flasks of confluent monolayers of secondary cultures were treated with 0.05% trypsin and 0.02% of EDTA in Puck's saline A (Gibco-Bio-cult Ltd., Scotland) according to the procedure used for subcultivation. After the cells had been dispersed, they were pooled and resuspended in a total volume of 140ml of growth medium. A viable cell count was made on it and 5ml of the suspension was seeded into each of 28 flasks (5×10^5 cell/25cm^2 flasks). The flasks were incubated at 36°C. Three flasks were removed at different intervals, the medium was decanted and the cells were treated with trypsin-EDTA mixture as for subcultivation. The cells were then suspended in 2ml of growth medium and a viable cell count was made on each sample in a Neubauer chamber. A count of the cells, in three flasks made 5 hours after seeding them indicated that the average efficiency of attachment of the cells to the substrate was 88%. The medium in the remaining flasks was decanted and replaced with 5ml of fresh medium. The growth curve of the cultures at 36°C over a period of 4 days is given in Fig. 11.

The curve shows there was an initial lag phase which lasted for about 16 hours. During this period, the cytoplasm of the cells extended; in all but the choroid epithelial cells, the cytoplasm assumed bi-polar and multipolar appearance. The rate of proliferation was gradual throughout the logarithmic phase. The curve levelled off between the 3rd and 4th day of incubation when the cell density was about 2.9×10^5 cell/cm^2.

Phase-contrast microscopy of samples before the cells were harvested and enumerated revealed that during the period of growth many telophase figures were dislodged from the substrate. Careful handling of the cultures did not prevent the figures from sloughing. Loss of dividing cells into the growth medium would account for the slow growth rate. Also, the irregular arrangement of the cells would arise from their varied morphology which in turn would restrict the terminal density of the cells, as well as contact inhibition. It is concluded that the growth rate of the cells was slow and doubling of the cell population did not take place. A possible explanation is the highly differentiated nature of some of the cells in this essentially mixed cell culture.

Fig. 11

GROWTH OF CULTURED ADULT RHESUS MONKEY BRAIN CELLS AT 36°C. AVERAGE CELL COUNT CALCULATED FROM HAEMOCYTOMETER COUNTS OF CELLS IN 3 FLASKS.



Karyotype Analysis

Chromsome studies were carried out on monolayers of tertiary cultures and on cells which had been in culture for 4 weeks. The unpublished method used is a modification of that described by Harnden (1974) for the preparation of human chromosomes from fibroblast cultures. The modified method (personal communication) is that of Miss Catherine M. Page of North London Polytechnic, Holloway, London.

0.5ml of an aqueous solution of colchicine (0.005% w/v) was added to the growth medium (5ml/flask) of 48 hours cultures which were in the log phase of growth. The cultures were incubated at 37°C for 1 hour. The tissue culture fluids were collected separately into conical plastic centrifuge tubes and the monolayers were treated with trypsin-EDTA mixture as for subcultivation. The cells were suspended in their respective tissue culture fluids and spun at 1000 rpm for 5 minutes. After discarding the supernatants, the packed cells were resuspended in hypotonic KCl solution (0.075M KCl diluted 3/4 in distilled water) and kept in a water bath at 37°C for 10 minutes. The suspensions were spun again for 10 minutes at 1000 rpm and the packed cells resuspended in a minimal amount of the hypotonic solution.

A drop of red blood cells delivered from a Pasteur pipette was added to the hypotonic solution to serve as a useful colour indicator during fixation. Sheep erythrocytes were used in this analysis. Addition of too many red cells makes it almost impossible to remove them from the final suspension. Also the cells tend to obscure mitotic figures in the final preparation of microscopic slides.

Freshly prepared Carnoy's fixative (3 parts of methanol and 1 part of glacial acetic acid) was added drop by drop to the cell suspension. In order to prevent the cells from aggregating during fixation, a drop of fixative was delivered on to the side of the tube held in a horizontal position. As the tube was tilted slowly into a vertical position, the bottom was tapped with a finger to keep the cells well dispersed and ensure thorough mixing of the fixative in the suspending fluid. The process was repeated several times. The cells were sufficiently fixed when the colour of the released haemaoglobin changed from red to dark or dirty brown. A large volume of fixative was added to the tube and the cells were spun down at 1000 rpm for 5 minutes. Each sediment was resuspended in fresh fixative in the same manner described above. The fixation process was repeated four times and finally the cells were allowed to stand at room temperature in fresh fixative for 30 minutes. They were sedimented and resuspended in a minimal volume of fresh fixative to yield a cell suspension with visible turbidity. A drop of this suspension was delivered from a Pasteur pipette on to a chilled, clean microscope slide and allowed to dry at room temperature. The dried spread was stained with diluted Giemsa (5ml of Giemsa (Raymond A. Lamb, Middlesex, London) to 100ml of Sorensen's phosphate buffer pH 6.8) for 3 minutes. The slides were rinsed in the buffer, dried, soaked in Michrome essence and mounted in Michrome.

Photographs of the stained preparations were taken under phase-contrast with a Zeiss Photomicroscope II on Ilford microneg film.

Out of the 100 metaphase plates which were counted, 97 of them had a normal male karyotype 42XY. Three cells had karyotype 41XY, 40XY and 41XY. The missing chromosomes were different in each case. This indicated cell breakage with subsequent loss of chromosome instead of a true chromosome abnormality. The karyotypes (Fig. 12) were arranged in four chromosome groups according to the classification of Fernandez-Donoso et al. (1970) and of De Vries et al. (1975)

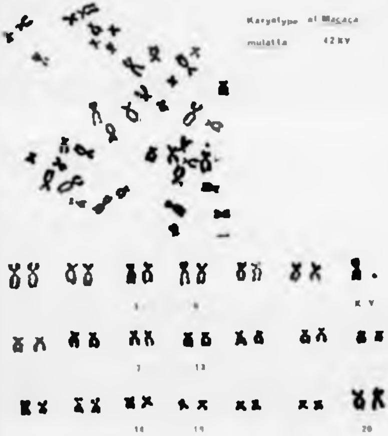


Fig.12 Chromosomes of normal cells from 4-week old cultures (5th passage) derived from adult rhesus monkey (macaca mulatta) brain cells.

4. VIRUS STUDIES

Viral infections of the central nervous system (CNS) may result as secondary complication of systemic infections caused by common viruses. A number of strains of Coxsackie and ECHO viruses, the arthropod-borne viruses, herpes simplex, mumps and lymphocytic choriomeningitis have been implicated in acute CNS diseases (Mayer *et al.*, 1960). The cellular population within the CNS is heterogeneous; such variability may reflect a selective susceptibility of the different cells to virus infection.

One of the mechanisms postulated on the mode of spread of viruses via blood-cerebrospinal fluid-brain, and blood-brain pathways considers a haematogenous invasion of virus to the nervous system. According to this mechanism during viremia virus in the blood might enter the cerebrospinal fluid (CSF) by either passing or growing through the choroid plexus. Pappas and Tennyson (1962) showed that capillaries within the choroid plexus have porous endothelium, and are surrounded by a loose stroma of connective tissue (Cancilla *et al.*, 1966). Suggestive evidence of CNS attack via the choroid plexus has been provided by Hamashima *et al.* (1959) who showed virus growth in the choroid plexus after subcutaneous inoculation of Japanese encephalitis virus in mice. Johnson *et al.* (1960) have suggested that in man, the growth of virus in the choroid plexus or leakage of virus into the CSF with initial growth in meningeal cells might explain the ready isolation of viruses such as Coxsackie and ECHO viruses from the CSF during infections of the central nervous system.

In this part of the project it was hoped that after the successful cultivation of tissue of the CNS obtained from adult rhesus monkeys, the mixed population of cells derived thereof might serve as host cells for virus replication and provide an *in vitro* evidence for the sensitivity of one type of cells over another to some of those viruses which cause neurological diseases. These can be divided into three categories. The viral encephalomyelitides and the encephalopathies include all those diseases with a specific viral aetiology. These diseases, which include rabies, poliomyelitis and the encephalitides caused by arboviruses such as equine, St. Louis, Japanese B and tick-borne encephalitis, have each characteristic pathological symptoms. The "slow" virus infections of the brain include Kuru and Creutzfeld-Jakob disease in man, Visna and Scrapie in sheep and mink encephalopathy. There is increasing evidence that subacute sclerosing panencephalitis may be caused by or associated with measles virus and acute necrotizing encephalitis may be caused by a herpes virus. A viral aetiology has also been suggested for von Economo's disease (lethargic encephalitis) which was once an important epidermic disease, and for progressive multifocal leucoencephalopathy, in which papovavirus like particles have been seen by electron microscopy.

Post infectious and postvaccinal encephalo-myelitis are similar inflammatory diseases (acute disseminated encephalomyelitis) which may follow a virus infection, particularly measles, chickenpox, smallpox and rubella, and also after influenza and infectious mononucleosis and the administration of live virus vaccines such as vaccinia and yellow fever. Immunization against rabies with the Semple and Fermi-type vaccines may result in a similar syndrome. The pathology of the disease is constant whichever virus is implicated but the virus cannot be recovered from the brain tissue or CSF. It is probably the result of an allergic or auto-immune type of reaction in the CNS.

A virus infection may also give rise to an inflammatory response in the meninges, a disease known as viral (or aseptic) meningitis. In addition to lymphocytic choriomeningitis other forms may occur during infection with poliovirus, various ECHO and Coxsackie viruses, mumps and herpesvirus. The virus may be recovered from the cerebrospinal fluid. Although the actual site of replication of these agents has not been finally established it is likely that the picornaviruses, for example, associated with aseptic meningitis, multiply in the meningeal cells. It has also been suggested, but as yet unconfirmed, that the viruses may destroy the cells of the blood vessels leading to secondary necrosis as a result of ischaemia.

There may be three possible mechanisms by which viruses cause neurological disease. First, many viruses give rise to an allergic response in the nervous tissue giving a disease which is constant whatever the causal virus may be. This occurs in postinfectious encephalitis. Second, some viruses may be capable of growing in the cells of the meninges, with or without cell destruction, inducing an inflammatory response, as in viral meningitides. And finally, a few viruses grow in the cells of the brain and spinal cord, usually with a cytopathic effect, each virus causing a disease with characteristic pathological changes. This occurs in poliomyelitis.

The naturally occurring neurotropic viruses may be exclusively neurotropic, that is, unable to grow in any tissue other than nervous tissue, e.g. rabies and some but not all of the "slow viruses". However, most can infect and grow in many tissues but have some property which enables them to infect and grow in nervous tissue. Sometimes the neurological involvement is a constant feature of the infection, for example, in the encephalitides due to arboviruses, and in other cases, the neurological involvement occurs only in a proportion of cases as in poliomyelitis. Finally, some viruses, which normally cause relatively mild illnesses in nature with only slight neurological involvement, have been implicated in acute neurological diseases, e.g. measles and herpes viruses. Whether these are due to virus mutation, or to individual physiological characteristics of the host, is not known. It is possible that some viruses causing meningitis may also grow in the brain tissue to give an encephalitis. These viruses would also be neurotropic.

In vitro studies have been done on the effect of neurotropic viruses on nervous tissue. Bunge and Harter (1969) described the fusion and destruction of some glial cells in cultures of mouse cerebellum by a tissue culture adapted strain of Visna virus. Neurons in these cultures were secondarily affected and dorsal root ganglion cultures were unaffected. Fernandez and Pomerat (1961) and Matsumoto and Yonezawa (1971) have described the destruction of neurons in puppy and kitten cerebellum cultures and in rat and mouse spinal ganglion cultures by both fixed and street strains of rabies virus.

The growth of cytopathology of herpes simplex virus in cultures of rat brain have been described by Feldman, Sheppard and Bornstein (1968), Mannweiler and Palacios (1969), and Leestma, Bornstein, Sheppard and Feldman (1969). Giant cells and typical inclusions were formed by glial cells but not by neurons. These latter degenerated non-specifically. Koestner, Kindig and Land (1970) have observed a similar effect on puppy brain cultures by a canine herpesvirus.

Studies on the effect of the encephalitic arboviruses on brain cultures have been carried out. Medearis and Kibrick (1958) reported the production of infective eastern equine encephalitis virus by suckling mouse brain cultures. Puppy and kitten cerebellum cultures have been used for the growth of Japanese encephalitis and Russian spring-summer encephalitis viruses (Hotta _et al_., 1967) and tick-borne encephalitis virus has been grown in human embryo cerebellum cultures (Mayer and Mitrova-Bellova, 1969). An inapparent infection of trypsinized suckling mouse brain cultures with Langat and Kyasanur Forest disease viruses has been reported by Illavia and Webb (1969).

Minced tissue suspensions of human embryo brain and spinal cord have been used for the growth of poliovirus (Sabin and Olitsky, 1936; Enders, Weller and Robbins, 1949) without cytopathic effect. But the destruction of neurons by poliovirus in human brain cultures was reported in 1955 and 1958 by Hogue _et al_.

Opere citato, the studies by Hogue and co-workers are the only one in which adult brain tissue as well as that of fetuses and infants were used. It was therefore pertinent to this project to study not only the growth of some viruses in our cultures derived from adult tissue but also to investigate whether neurovirulence of some could be enhanced or conferred upon the virus progeny produced by the cells.

A) ECHOVIRUS TYPE 11

The echoviruses (enteric cytopathogenic human orphan viruses) are a group of infectious agents of the human intestinal tract. They have been placed in the enterovirus subgroup of the picornavirus family and they share certain physical, biological and chemical characteristics, as well as epidemiological patterns, with the coxsackieviruses, polioviruses and rhinoviruses. Each of these subgroups is distinct. Echovirus prototypes are numbered 1 to 33 but three of these, 8, 10 and 28, have been removed from the classification group. Types 1 and 8 have been put together as type 1 because they are closely related (Committee on Enteroviruses, 1962). Echovirus type 10 has been removed from the enterovirus group because of its large size (70nm) and unique architecture of 92 capsomeres compared with 32 for the picornaviruses. Echo 10 has been reclassified as reovirus 1 (Sabin, 1959a). Echovirus 28 is now the first member of the rhinovirus subgroup. It is of the same size and architecture as the enteroviruses. However, it has been classified as a rhinovirus because it is isolated from the respiratory tract and is acid labile.

The prototype of echovirus type 11 (Gregory Strain) was first isolated from rectal swabs from children in Cincinnati, Ohio, and Mexico City, Mexico, who had no clinical illness (Ramos-Alverez and Sabin, 1956). Echovirus 11 has also been associated with aseptic meningitis (Elvin-Lewis and Melnick, 1959; Von Zeipel et al., 1960), severe and mild paralytic diseases (Steigman and Lipton, 1960), exanthematous diseases (Cherry et al., 1963) and acute gastroenteritis (Klein et al., 1960). Echovirus type 11 has been implicated in some cases of upper respiratory disease, characterised by coryza, sore throat, cough and slight fever, among children. Seven isolates of the virus were obtained from 36 children during an outbreak of respiratory illness in a day nursery. All the isolates but one were recovered from faecal material (Philipson, 1958). In an outbreak of respiratory disease involving seven newborn infants and five mothers, 6 infants and three mothers were clinically ill and from these, four agents were isolated. Two were echovirus type 11, one was echo 18 virus and one was coxsackie A9. Four mothers and three of their children had serum antibodies against echovirus 11 (Berkovich and Kibrick, 1964). Association between echo 11 virus and children with non-diptheric croup has also been reported. The virus was isolated from 32% (17/53) of children with the croup syndrome and 12% (8/67) of children in an unmatched control group. In the children with croup, virus was recovered more frequently from the throat than from faeces (Philipson and Wesslen, 1958; Philipson, 1958).

Echovirus type 11 is 28nm in diameter as determined by gradocol filtration (Soloviev et al., 1967). Electron microscopic studies of particles purified by density-gradient centrifugation in CsCl and stained with phosphotungstic acid reveals a polyhedron with icosahedral symmetry and 32 capsomeres (Mayor and Melnick, 1962). Thermal inactivation studies at 56°C show that loss of infectivity of echovirus 11 follows a first-order reaction in which the virus becomes non-infectious after 15 minutes. In glycerol, the rate is not first order and the virus remains infectious after 2 hours. Virus haemagglutinin is undetected after 15 minutes. At 37°C, a first-order inactivation rate is observed for both untreated and glycerinated virus. The virus retains its infectivity after 80 hours and the haemagglutinin is destroyed in 120 hours. If the virus is suspended in a glycerol-containing medium, infectivity is preserved (25°C for 6 days; 4°C for 2 months and for 12 months at -20°C) (Philipson and Wesslen, 1958; Philipson, 1958). Wallis and Melnick (1962) have reported that echoviruses in general are protected from thermal inactivation by molar $MgCl_2$ and other salts of divalent cations (50°C for 1-2 hours; 37°C for 1-3 days; 20°C for 1-3 weeks and 4°C for 1-2 years).

Echovirus type 11 is stable when exposed to acid (pH 3.0) at 24°C. At pH 5.0-8.0, it is stable for 10 hours at 37°C, with an inactivation rate which suggests a first-order reaction. It is rapidly inactivated at 37°C, below or above this pH range. The density of the virus is 1.3 g/cm^3 and its sedimentation constant is 113S and 105S.

Echovirus 11, like other members of the picornavirus group, contains a core of RNA and the isolation of infectious RNA from this virus has been reported by Wenner (1962). The protein content of the virus has not been characterised and neither has any enzyme been described. Unlike, for example, arboviruses, and myxoviruses, echoviruses retain their infectivity after exposure to 20% diethyl ether for 18 hours at 4°C (Philipson, 1958). It is inactivated by formaldehyde. However, echoviruses in general are insensitive to 70% alcohol, deoxycholate and various detergents which inactivate other viruses (arboviruses etc.).

All echoviruses but types 22 and 23 are inhibited from propagating in cultures by 2-(α-hydroxybenzy)-benzimidazole (HBB) (Eggers and Tamm, 1961) and by guanidine whose inhibitory action on enterovirus synthesis in cell cultures was first reported by Rightsel *et al.*, (1961). These two drugs have been shown (Eggers and Tamm, 1963a) to exhibit a synergistic action of inhibiting the appearance of viral RNA polymerase and the synthesis of both viral RNA and viral protein.

Echo 11 virus grows in a number of cell cultures. Susceptible cells include primary human embryonic lung, human kidney, rhesus monkey kidney and human amnion (Ramos-Alverez and Sabin, 1956; Buckland *et al.*, 1959 and Hsuing, 1962). Growth of virus in cell cultures leads to cytopathic effect which consists of accumulations of small refractile round cells that lose their dense appearance and slough from the glass and infected cells stained with Giemsa showed no inclusion bodies (Philipson and Wesslen, 1958). Viral replication occurs in the cytoplasm of the infected cell. Soloviev *et al.* (1967) have described that, in infected monkey kidney cells, there was an alteration of the chromatin pattern with the formation of transitory small intranuclear inclusions, eosinophilic and basophilic intracytoplasmic inclusions, and the release of basophilic inclusions from the cells.

For primary isolation, HeLa, Detroit-6, Patas monkey kidney and HEp-2 cells are not susceptible, however Philipson and Wesslen (1958) and Hsuing (1962) have reported that the virus can be adapted to these cell cultures.

In addition to infective virus, echoviruses produce complement fixing and haemagglutinating antigens. Only human type O erythrocytes have receptors specific for echovirus haemagglutinin. Philipson and Choppin (1960) have suggested that this haemagglutinin is probably dependent on intact sulphydryl groups of the viral protein. Both the infectious particle and the haemagglutinin participate in the haemagglutination reaction and the conclusion has been drawn by Philipson and Wesslen (1958) and Philipson (1958) that the two are probably identical - ultracentrifugation does not separate the haemagglutinin from the infective virus. Echo 11 infective particle and haemagglutinin absorbs onto erythrocytes more readily at 4°C than at 37°C and elution occurs within 6 hours (Philipson, 1958). It has been reported by Leers (1969) that incubation with chloroform had no effect on the haemagglutinin of echovirus type 11.

EXPERIMENTAL

Echovirus type 11 (prototype strain Gregory) was obtained from the Virus Reference Laboratory, Central Public Health Laboratory, Colindale, London. The virus had been passaged seven times in rhesus monkey kidney cultures. After two passages at 36°C of the virus in cultures derived from adult rhesus monkey brain, stock virus was prepared from infected cultures. These had been infected for 48 hours and showed extensive cytopathic changes in the cells. The virus was harvested by freezing and thawing infected cultures three times and the supernatant fluid was centrifuged at 3000 rpm for 15 minutes to remove cellular debris. The supernatant was stored in glass vials at -50°C.

Infectivity titre of the stock virus was determined by titration in test-tube cultures derived from rhesus monkey brain cells. The tubes had been seeded with 1×10^5 cells/ml/tube 2-3 days before use. Serial ten-fold dilutions of stock virus were prepared in maintenance medium. The growth medium in the tubes was removed and 0.1ml of each dilution was inoculated into each of four replicate tubes. The virus was allowed to adsorb for 1 hour at 36°C and without washing 0.9ml of maintenance medium was added to the inoculum in each tube. After 3 days incubation at 36°C, the tubes were scored for cytopathic changes. The titre in $TCID_{50}$/ml was estimated by the "moving average" method of Thompson (1947).

The identity of the virus was confirmed by two tests: 1) serum neutralisation test using inactivated rabbit anti-ECHO 11 neutralising serum. The procedure has been described under serological methods, 2) haemagglutination-inhibition test with high-titred monkey neutralising serum. Human group O cells were used for HA and HAI tests which were performed in a combined form by chess-board titration. This method has been found useful when only one antiserum is to be tested. Also errors involved in the preparation of virus dilutions would be constant over the whole test. Furthermore, a considerable length of time was saved in this method over the standard method in which the test is conducted in two steps. This was achieved, however, at the expense of the volume of antiserum required for the test. But it was thought that the practical use of the chess-board method which has hitherto been applied only to complement-fixation tests could equally be applied to HA and HAI tests particularly in the teaching of students.

The basic plan of the test is a two-dimensional array of the two reactants-antigen and antiserum in WHO haemagglutination plates. 0.2ml amounts of varying dilutions of the antigen in normal saline were distributed into each of the wells in the vertical columns. Each well in the last column received 0.2ml of the diluent. Equal volumes of two-fold dilutions of the antiserum were delivered into the wells in rows and 0.2ml of saline to each of the wells in the last row. After the two reactants had been allowed to react, 0.1ml volumes of 1% suspension of human O cells in saline were distributed into the wells.

The last row would formally represent the standard HA test. The titre of the antigen (IHAU) would be the highest dilution which gave complete agglutination in this row. Wells in the last column would determine the degree of non-specific agglutinins in the serum if these had previously not been absorbed with the blood cells used in the test. The titre of the serum (highest dilution) which produced complete inhibition of agglutination with any number of HA units would be obtained in the well common to both the chosen vertical column and the corresponding row.

The monkey anti-ECHO 11 serum used for the test was inactivated at 56°C for 30 minutes. It was then absorbed with 10% human O cells in the serum at room temperature for 1 hour. The cells were sedimented by centrifugation at 3000 rpm for 10 minutes and the supernatant serum was collected. Monolayers of cells derived from rhesus monkey brain in three Falcon flasks were scraped with a rubber policeman into the growth medium. The suspensions were pooled and centrifuged at 1000 rpm for 10 minutes. The supernatant tissue culture medium was discarded. The cells were suspended in the antiserum and left at +4°C overnight. It was finally centrifuged at 3000 rpm for 10 minutes and the serum collected for the test.

A concentrated suspension of virus was prepared by ultracentrifugation at 30,000 rpm for 1 hour. The pellet of virus particles was resuspended in one-fifth of the original volume of medium.

In the test proper, mixtures of antigen and antiserum were allowed to react at room temperature for 1 hour before the red blood cells were added. The results were read after an hours incubation at room temperature.

The growth of ECHO 11 virus at 36°C was determined in monolayers of secondary cultures derived from brain cells in 25cm^2 Falcon flasks. After 48 hours growth when the cultures were still in the log phase, cells in five random samples of the cultures were trypsinised and suspended in 2ml of medium. A viable count was made on each sample in a Neubauer chamber. An average number of 6.6×10^5 cells per flask was obtained. Cultures were inoculated with 1ml of diluted suspension of stock virus containing 2.1×10^6 TCID$_{50}$/ml. After adsorption at 36°C for 1 hour, each culture was washed with three changes of 5ml of maintenance medium. Two ml of the medium was delivered into each flask and incubated at 36°C. At 2 hourly intervals during a 24 hour period, one flask was removed and the harvested medium was stored at -50°C to be assayed later for extracellular virus. Two ml of sterile PBS was delivered into each flask and the monolayer was scraped with a rubber policeman. The infected cell suspension was stored at -50°C. At the end of 24 hours all infected cell suspensions were harvested for intracellular virus by repeated freezing in acetone-solid CO_2 mixture and thawing. Infectivity titrations for extr and intra-cellular virus were done as for the procedure described above. Three uninoculated control monolayers were harvested after 24 hours incubation and similarly treated.

RESULTS

Examination by fluorescence microscopy of acridine orange stained preparation of infected Leighton tube cultures showed marked cytopathic effect within 24 hours. The effect consisted of groups of small, round cells the nuclei of which had condensed into ill-defined structures surrounded by retracted cytoplasm. These cells fluoresced pale orange. The cells in uninoculated control cultures which were similarly stained fluoresced metachromatically - cytoplasm fluoresced orange to brick-red and the nucleus, yellowish-green.

The results of the neutralisation titrations of rabbit anti-ECHO 11 serum against 10^3TCID$_{50}$ of stock virus are given in Table 4 . The highest dilution of serum which completely inhibited the cytopathic changes produced by 10^{-4} dilution of virus control in 3 days was 1/200

Table 4 . Neutralisation Test: Stock ECHO 11 virus vs Homologous antiserum

Serum dilution	CPE after 3 days	Virus dilution	Control CPE
1:25	0/4	10^{-4}	4/4
1:50	0/4	10^{-5}	4/4
1:100	0/4	10^{-6}	4/4
1:200	0/4	10^{-7}	2/4
1:400	4/4	10^{-8}	0/4
1:800	4/4	10^{-9}	0/4

Numerative is the number of tubes showing CPE
Denominator is the number of tubes inoculated

Table 5 shows a typical set of results of HA and HAI tests by the chess-board method. 4HA units were inhibited by 1:1600 dilution of the antiserum.

Table 5 . HA and HAI tests of Echovirus Type 11 (5x conc) and Homologous monkey antiserum in a chess-board experiment

		Antigen Dilutions (1:)							
		10	20	40	80	160	320	640	C
Antiserum Dilutions (1:)	100	-	-	-	-	-	-	-	-
	200	-	-	-	-	-	-	-	-
	400	-	-	-	-	-	-	-	-
	800	-	-	-	-	-	-	-	-
	1600	+	±	-	-	-	-	-	-
	3200	+	+	+	+	-	-	-	-
	6400	+	+	+	+	±	-	-	-
	C	+	+	+	+	±	-	-	-
		16	8	4	2	1 HAU			

+ : Complete agglutination
± : Partial agglutination
- : No agglutination

A numerical example of the estimation of $TCID_{50}$ by the Thompson's "moving average) method is illustrated below with the results of the infectivity titration of the stock virus.

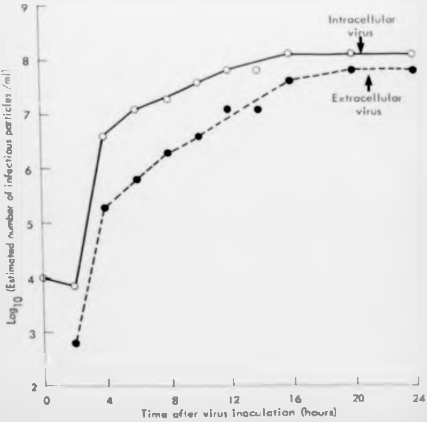
The echovirus growth cycle was determined in test-tube cultures derived from brain cells by infectivity titrations. The results graphically depicted in Fig. 13 showed that the eclipse phase for the echovirus was less than 4 hours, that intracellular growth of virus was exponential and completion of replication required a minimum of 16 hours after which the growth levelled off. Release of infectious virus into the extracellular fluid occurred simultaneously after the eclipse phase and increased exponentially up to 20 hours after infection. Approximate one-step growth curve conditions were obtained by infecting the culture with a large inoculum, which should theoretically leave no uninfected cells. An attempt was made to remove the unadsorbed virus by washing. However, some virus from the inoculum clearly remained.

Three monolayer cultures inoculated in parallel with those for the growth curve studies were examined by phase-contrast microscopy to follow the progression of CPE. The onset of cytopathic changes was evident 6 hours following infection. These changes were observed primarily as foci in the islands of choroid epithelial cells. At 48 hours following infection when most of the cells had come off the flask, the apparently normal cells still attached to the surface consisted mainly of spindle-shaped and multipolar (glial) cells. The infected tissue culture medium was removed, the cells were washed thrice and 3ml of maintenance medium was delivered into each flask. After further incubation at 36°C, these cells eventually degenerated on the 8th day after infection with the characteristic pattern observed 7 days earlier.

Assuming any cell type in the cultures could be infected syndronously duirng the one hour adsorption period, the tempting conclusion would be that the choroid plexus epithelial cells succumbed to infection sooner than the other cell types. If, on the other hand, there was a variation of susceptibility within the mixed cell types, it would be reasonable to suggest that the chloroid epithelial cells are more susceptible to infection than the morphologically

Fig. 13

GROWTH CYCLE OF ECHOVIRUS TYPE II (PROTOTYPE STRAIN GREGORY) AT 36°C IN ADULT RHESUS MONKEY CULTURE DERIVED FROM THE BRAIN.



different cell types.

Measurements of the particles were determined from electron micrographs of negatively stained preparations. A suspension of stock virus was spun down at 30,000g for 2 hours in a Sorvall OTD-2 ultracentrifuge. The pellet was stained with 2% ammonium molybdate pH 6.3 and examined in the electron microscope (AEI EM 801). The particles consisted of full and empty capsids (Fig. 14). The average diameter of the particlex was 31.62nm and the central area occupied by the core measured 21.60nm.

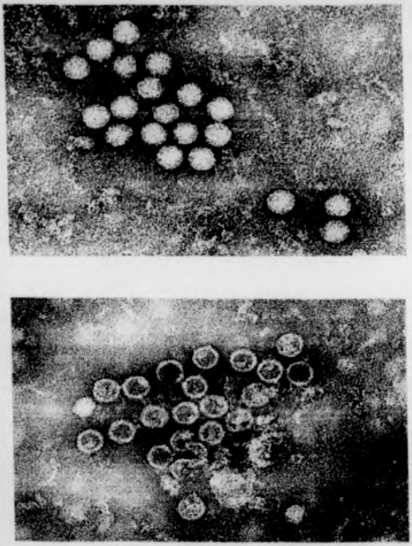


Fig. 14 : Echo 11 virus (Prototype strain Gregory) negatively stained with 2% Ammonium molybdate.

Above : A group of virus particles complete with core.

Below : Empty capsids from the same preparation as above. x 289,800

(B) REOVIRUS I

The reovirus (respiratory enteric orphan) group, which comprises types 1, 2 and 3 was for some years classified as echovirus 10 (Sabin, 1959). All the three types were initially isolated from rectal swabs from children and most strains of subtype 1 have been isolated from healthy individuals. The King strain of reovirus 1 was associated with fever and the NS-111 strain with coryza, and Hull et al. (1956) isolated a reovirus type 1 (SV_{12}) of simian origin.

Reoviruses are distinguishable from enteroviruses, myxoviruses, respiratory syncytial virus, adenoviruses, and some simian viruses by size, type of cytopathic effect, resistance to ether, haemagglutination and serology. There is no antigenic relationship between reoviruses and coxsackie A7, 9, 11, 13, 14, 15 or 18; coxsackie B1 - 5; echovirus 11 and echovirus 1 - 9.

Jordan and Mayor (1962), using electron microscopy, calculated the mean diameter of reovirus type 1 to be 60nm. A diameter of 75nm has been reported for a strain of reovirus type 1 (Rhim et al., 1962). Reoviruses are icosahedral in shape, with a capsid composed of 92 elongated hollow capsomeres (Jordan and Mayor, 1962). The calculated dimensions for the capsomeres of type 2 are 11.6nm by 11nm (Loh et al., 1965). Type 3 has been shown to be an icosahedron with a 5:3:2 symmetry. The particles have an inner core with subjacent shell, the capsomeres measured 10nm by 8nm and a central hollow core of 4nm in diameter (Dales and Gomatos, 1965). By the use of electron microscopy, Rhim et al., (1961) found two types of virus particles, usually in crystalline array and also association of infectivity with complete rather than coreless viral particles. Mayer et al., (1965) found that coreless particles increased in number with the time of incubation in the cell culture.

Complete inactivation of reoviruses occurs at 56°C within 45 minutes but not after 30 minutes. At 37°C, degradation of the infectivity of reovirus 1 is complete in 30 days; at 4°C, infectivity is detectable after 2 months (Usmankhodzhayer and Zakstelskaya, 1964). A strain of type 1 has been found to have a half-life of 0.79, 2.0 and 3.7 days at 37°C, 24°C and 4°C respectively (Rhim and Melnick, 1961). An increase of infectivity titres has been obtained by heating reovirus 1 at 50°C to 55°C for 5-15 minutes in the presence of $MgCl_2$; no effect of HA was found but the progeny of heat-resistant viruses were susceptible to cold (Wallis, 1964).

Reoviruses are not inactivated at pH 3.5 and are relatively stable over a wide range of pH (Ketler *et al.*, 1962). Reovirus RNA has a sedimentation coefficient of 11 - 15S, a mean buoyant density of 1.61 g/cm^3 in Cs_2SO_4, and a molecular weight of approximately 10^7 daltons. Thermal denaturation begins at 78°C and is complete at 85°C (Gomatos and Stockenius, 1964; Iglewski and Franklin, 1967). The density of the infectious virion has been determined as 1.37g/cm^3 and haemagglutinating activity was found in a band with a density of 1.29g/cm^3 (Fouad and Engler, 1966; Engler and Fouad, 1967).

The RNA content of reoviruses was determined with the Feulgen stain which gave a negative reaction, and Unna - Pappenheim stain after treatment with ribonuclease (Sabin, 1959). The oligonucleotides which make up the RNA are heterogeneous in both length and base composition. It has been calculated that about 2,000 lengths of these oligonucleotides are present in each virion and the majority of low molecular weight RNA is in lengths of 6 to 12 nucleotides (Bellamy *et al.*, 1970 and Bellamy and Hole, 1970). Electron microscopic studies by Dunnebacke and Kleinschmidt (1967) yielded values for the length of RNA filaments ranging from 1 to 7.7um. The RNA, which constitutes about 14% - 15% of the virion, has been shown for type 3 to have compact, double-stranded nucleic acid. Single-stranded RNA of reovirus has a base composition of 88% adenine, 10.5% uracil, 1.5% cytosine and no detectable guanine. The strand can be broken into two fragments of which one is susceptible and the other resistant to RNase. The RNA does not hybridise with double-stranded RNA (Bellamy and Joklik, 1967; Joklik and Bellamy, 1969).

The virion contains an RNA-dependent RNA polymerase that transcribes virus-specific RNA from the double-stranded RNA genome. This polymerase, or transcriptase, requires all four ribonucleoside triphosphates and divalent cations. The system is not inhibited by actinomycin D or 5-bromocytidine-5'-triphosphate but is inhibited by p-chloromercuribenzoate (Kapuler, 1970).

Studies by gel electrophoresis have shown that the capsid protein of reoviruses is composed of seven species of polypeptides of which most of the major and all the minor components are synthesized early in the infectious cycle (Loh and Oie, 1969). Of the capsid polypeptides which have been found in virus-infected cells, (μ_2) is the major constituent of the virion. Five other capsid polypeptides ([illegible], σ_2 and σ_3) are found in good amounts and one (σ_1) is in small quantities. These polypeptides are synthesized synchronously (Zweerink and Joklik, 1970).

The lack of essential lipids in the structure of reoviruses has been demonstrated by the retention of infectivity when they are treated with 20% diethyl ether at 4°C for 18 hours (Rosen *et al.*, 1960). Chloroform, however, reduces their infectivity and inactivates the haemagglutinin (Rozee and Leers, 1967). The treatment of reovirus type 1 with proflavine resulted in reduced HA activity and elimination of cytopathogenicity and cultures inoculated with proflavine-treated reovirus are refractory to superinfection by other viruses (Zelan and Labzoffsky, 1965).

Reoviruses are resistant to 2% lysol, 3% formalin and 1% H_2O_2 when incubated for 1 hour at room temperature. They are completely inactivated by 70% ethanol (Stanley *et al.*, 1953).

Splondore and Schaffer (1965) have shown that incubation of reovirus type 1 with trypsin resulted in an increased titre of infectivity. Incubation of reoviruses, in general, with chymotrypsin results in an increased titre of infectivity due to the production of subviral particles that catalyse the synthesis of polynucleotides (Levin *et al.*, 1970). Wallis *et al.* (1966) also demonstrated that the addition of pancreatin to reovirus-infected cells resulted in enhanced viral replication.

All three types of reovirus are insensitive to the inhibitory action of hydroxybenzyl benzimidazole (HBB) (Egger and Tamm, 1961). Reovirus type 1 is resistant to the action of guanidine (Rightsel *et al.*, 1961).

Reoviruses can be sedimented by ultracentrifugation at 6000g in 10 minutes. All three serotypes of reovirus, when cultivated in rhesus monkey kidney cell culture, agglutinate human group O erythrocytes; the exception being type 3 which also agglutinates ox erythrocytes. Newlin and McKee (1966) found that type 3 infected cultures yielded two virus populations, one which agglutinated human and ox erythrocytes and the second only human erythrocytes. One HA unit of reovirus type 1 is equivalent to 6.2×10^6 pfu/ml (Eggers *et al.*, 1962). The haemagglutinin and infectious particle are produced synchronously but the release into tissue culture fluid proceeds at a very slow rate. Incubation with trypsin increases the titre of haemagglutinin of reovirus 1 and the action of potassium periodate on all three types of reoviruses causes a decrease in the HA titre. Because of the inhibitory action of capsid-bound sugar, N-acetyl-D-glucosamine, it has been postulated that the haemagglutinin is a glycoprotein (Gelb and Lerner, 1965). At 37°C, infectivity is readily lost while the HA titre hardly changes for eight months. The inference here is that the infectious particle and the haemagglutinin are separate entities; however, when all three serotypes are exposed to chloroform, the haemagglutinins are destroyed and only a reduction in infectivity occurs. (Zalan and Labzoffsky, 1967; Leers and Rozee, 1968). Natural inhibitors of haemagglutination have been found in the serum of a host of animals including mice, rats, rabbits, guinea pigs, chimpanzees, rhesus and grivet monkeys, cattle, horses, swine, dogs and cats. Removal of heat-stable inhibitors have been achieved by treatment with kaolin, chymotrypsin, phospholipase C or incubation with rivanol. Tissue culture harvests of normal and infected cells have been found to contain HA inhibitors which can be destroyed by proteolytic enzymes and organic solvents. These inhibitors are considered to be lipoprotein (Schmidt et al., 1964).

All three types of reovirus share a common antigen which is detectable by complement fixation. The serotypes possess one and possibly two group antigens in addition to one-type specific antigen (Leers et al., 1968). They can be distinguished by neutralisation and tests by haemagglutination-inhibition. Isolates from human and animals are antigenically indistinguishable (Rosen, 1962). Rabbits, guinea pigs or roosters develop CF, HAI and neutralising antibody after inoculation with harvests of infected cultures. Gomatos et al. (1962) have found from their studies that the neutralising and HAI antibodies are homotypic for a serological subtype with slight heterotypic reaction.

A number of tissue culture systems permit the growth of reoviruses. These include primary rhesus monkey kidney cultures, HeLa cells and human amnion cells (FL). Ensminger and Tamm (1969) have reported that infection of monolayers with reoviruses resulted in 80% inhibition of cellular DNA synthesis but protein synthesis was unimpaired. A retention of the structural integrity of the host DNA was also observed. However, when suspension cultures were used, there was a 30% - 40% inhibition of host protein synthesis. Growth of reoviruses in cell cultures leads to a characteristic cytopathic effect which is different from that produced by picornaviruses. Infected cells separate from the sheet and assume a granular, degenerative appearance with an intact nucleus. They do not come off the glass readily but often remain attached to the glass by a single process and flutter in the medium during microscopic examination. Infected cultures stained with haematoxylin-eosin or Giemsa reveal intracytoplasmic inclusion bodies which give a negative staining reaction with Feulgen or mucopolysaccharide stains. The bodies also fail to stain red by the Unna-Pappenheim method after treatment with ribonuclease (Sabin, 1959). Antigenic material and viral RNA develop in the cytoplasm (Spendlove et al., 1963) and can be demonstrated by fluorescent antibody staining.

EXPERIMENTAL

The Lang prototype strain of reovirus type 1, was supplied by the Virus Reference Laboratory, Central Public Health Laboratory, Colindale, London. The virus was passaged twice in cultured cells derived from the brain of adult rhesus monkey at 36°C before stock virus was prepared from infected cultures. The infected medium in 25cm^2 Falcon flask cultures was replaced with fresh medium when CPE was first observed in the cultures. The collected fluid was frozen at -50°C. The medium was replaced frequently to prevent heat inactivation of virus released from degenerated cells. After 8 days the cells were harvested and pooled with the collected fluids. The suspension was frozen and thawed three times and clarified by centrifugation at 3000 rpm for 10 minutes.

The supernatant was divided into two volumes. One half was frozen at -50°C to be assayed by quantal response in test-tube cultures. The other volume was ultracentrifuged at 35,000 rpm for 2 hours. The pellet was resuspended in PBS to a sixth of the original volume. This concentrate was used for complement-fixation tests.

The titration of the stock virus was done in the same manner as for ECHO 11 virus. The titre in $TCID_{50}$/ml was estimated by Thompson's "moving average" method. Inactivated rabbit anti-Reo 1 serum was used for neutralisation and complement fixation tests. In the neutralisation test, ten-fold serial dilutions of the stock virus were titrated against 1/200 dilution of the antiserum.

The growth curve studies of reovirus type 1 (Lang prototype strain) at 36°C were done in monolayers of secondary cultures derived from brain cells. The cultures were infected with 1ml of diluted stock virus (passage 3) in maintenance medium at an estimated multiplicity of infection of 1:1. After adsorption at 36°C for 3 hours, the inoculum was pipetted off and the monolayers were washed with three changes of maintenance medium. The flasks were re-incubated at 36^{0}C after each had received 2ml of medium. During the incubation period of 4 days, samples were removed at intervals and the infected cells together with the medium was harvested by freezing and thawing. When all the samples had been obtained, the cell-free extracts were assayed for total virus by complement-fixation test. For the test, varying concentrations of guinea-pig complement were titrated against 1:10 dilution of the antiserum and undiluted test samples. Cell-free extract of uninfected cultures was used as antigen control. The procedure is described under serological methods The logarithms of the number of units of complement fixed were plotten against the time at which samples were obtained.

Leighton tube cultures of cells derived from the brain were infected with diluted stock virus for microscopic study of the growth of the virus. Infected and control cultures were stained by the indirect fluorescent antibody technique at different times after infection.

RESULTS

The titre of the stock virus (passage 3), calculated by the "moving average" method, was 1.78 x 10^{6} TCID$_{50}$/ml. 100 TCID$_{50}$'s/ml of the stock virus were neutralised with 1:200 rabbit anti-Reovirus type 1 serum. The results of the neutralisation test are given in Table 6 below.

Table 6 . Neutralisation Test using [illegible] dilution of immune serum titrated against stock virus (passage 3)

TEST		VIRUS CONTROL	
Virus dilution	CPE after 5 days	Virus dilution	CPE after 5 days
10^{-1}	4/4	10^{-3}	4/4
10^{-2}	4/4	10^{-4}	4/4
10^{-3}	4/4	10^{-5}	4/4
10^{-4}	3/4	10^{-6}	4/4
10^{-5}	0/4	10^{-7}	1/4

Numerator: No. of tubes showing CPE
Denominator: No. of tubes inoculated

The electron micrograph (Fig. 20) shows a group of particles of reovirus type 1 (Lang strain). The particles prepared from the stock virus suspension, were negatively stained with 2% ammonium molybdate pH 6.3. The average diameter of the particles was 79 nm and the shell measured on average 17nm.

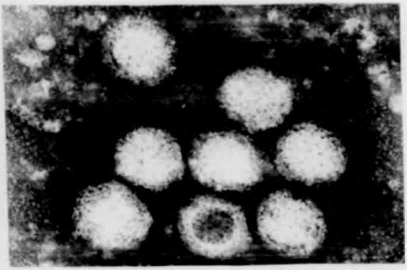


Fig. 15. Electron micrograph of a group of Reovirus type 1 (Lang strain) showing hexagonal outline. Capsomeres are visible at the periphery of the particles. One empty capsid. Negatively stained with 2% ammonium molybdate. x 289,800

The graphical representations of the log units of complement fixed by antigen-antibody complexes in the complement fixation test are shown in Fig. 16 . The data of the test are given in Table 7 . The antigen (or antiserum) contours represent the profile of the reaction between an antigen (or antiserum) dilution and varying concentrations of antibody (or antigen) and of complement. The one-unit complement contour in Fig.17 represents mixtures of antigen and antibody dilutions which would fix one unit of complement. The points on the curve were derived from the intersections of antigen contours and antiserum contours with the base line 0.0 log (i.e. 1 unit of complement fixed). The points on the complement contour derived from the antiserum contours are indicated by a full circle (●) and those from antigen contours are indicated by a full triangle (▲).

From the one-unit complement contour, the maximum antiserum titre is 1:32 and the maximum antigen titre of the stock virus (passage 3) is 1:246. The estimated antigen titre is 1:241 i.e. 0.1038μl of antigen contained in 25μl of diluted antigen. The 95% confidence limits are

0.1038 ± 0.03917

i.e. 0.0646 - 0.1430μl

The method for estimating the titre and its standard error adapted from Elston (1964) is given under serological method. A numerical example of the method is given below using the data of the complement-fixation test.

Fig. 16

TITRATION OF REOVIRUS TYPE I (LANG PROTOTYPE STRAIN) WITH RABBIT REOVIRUS I NEUTRALISING ANTISERUM. DATA FROM TABLE 7

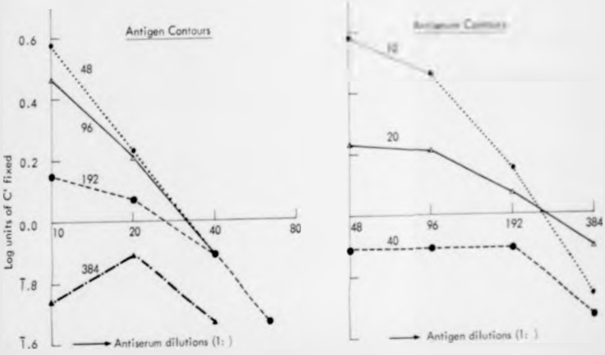
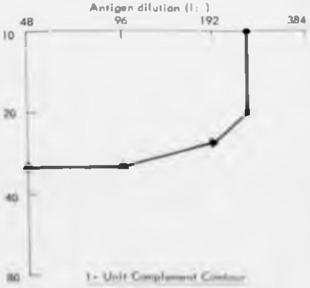
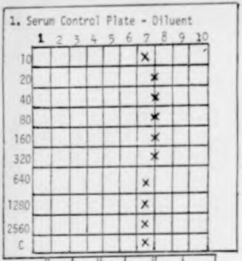


Fig. 17

TITRATION OF REOVIRUS TYPE I (LANG PROTOTYPE STRAIN) WITH RABBIT REOVIRUS I NEUTRALISING ANTISERUM. POINTS DEFINING THE COMPLEMENT CONTOUR ARE OBTAINED FROM THE INTERSECTIONS OF ANTIGEN AND ANTISERUM CONTOURS WITH THE BASE LINE AT 0.0 LOG UNITS OF COMPLEMENT (1 UNIT OF C FIXED.




1. Serum Control Plate - Diluent

	x	u					
1	10	0·13					
2	20	0·10					
3	40	0·10					
4	80	0·10					
5	160	0·10					
6	320	0·10					
7	640	0·13					
8	1280	0·13					
9	2560	0·13					
10	X =	0·13					

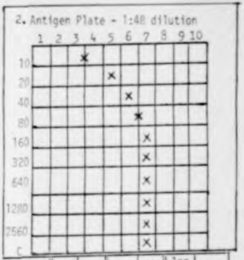
Table 7 a

Complement fixation of Reovirus type 1 (Lang) antigen with rabbit immune serum. In each row the cross indicates the column in which the 50% end-point was observed. Data for figs. 16, 17.

$k = 0.13$

$\log k = \bar{1}.11$

$w = \log f - \log k$


2. Antigen Plate - 1:48 dilution

	x	z	u	z−u	f	log f	w
1	10	0·62	0·13	0·49	0·49	$\bar{1}$·69	0·58
2	20	0·31	0·10	0·21	0·21	$\bar{1}$·34	0·23
3	40	0·20	0·10	0·10	0·10	$\bar{1}$·00	$\bar{1}$·89
4	80	0·16	0·10	0·06	0·06	$\bar{2}$·78	$\bar{1}$·67
5	160	0·13	0·10	0·03	0·03	$\bar{2}$·48	$\bar{1}$·37
6	320	0·13	0·10	0·03	0·03	$\bar{2}$·48	$\bar{1}$·37
7	640	0·13	0·13	0		∞	
8	1280	0·13	0·13	0		∞	
9	2560	0·13	0·13	0		∞	
10	v =	0·13	0·13	0		∞	

Table 7.3

3. Antigen Plate - 1:96 dilution

	1	2	3	4	5	6	7	8	9	10
10				X						
20					X					
40						X				
80							X			
160							X			
320							X			
640							X			
1280							X			
2560							X			
C							X			

	x	z	u	z-u	f	log f	w
1	10	0·50	0·13	0·37	0·37	$\bar{1}$·57	0·46
2	20	0·31	0·10	0·21	0·21	$\bar{1}$·32	0·21
3	40	0·20	0·10	0·10	0·10	$\bar{1}$·00	$\bar{1}$·89
4	80	0·13	0·10	0·03	0·03	$\bar{2}$·48	$\bar{1}$·37
5	160	0·13	0·10	0·03	0·03	$\bar{2}$·48	$\bar{1}$·37
6	320	0·13	0·10	0·03	0·03	$\bar{2}$·48	$\bar{1}$·37
7	640	0·13	0·13	0		∞	
8	1280	0·13	0·13	0		∞	
9	2560	0·13	0·13	0		∞	
10	$\bar{x}$ =	0·13	0·13	0		∞	

4. Antigen Plate - 1:192 dilution

	1	2	3	4	5	6	7	8	9	10
10					X					
20										
40						X				
80							X			
160							X			
320							X			
640							X			
1280							X			
2560							X			
C							X			

	x	z	u	z-u	f	log f	w
1	10	[illegible]	0·13	0·18	0·18	$\bar{1}$·26	0·15
2	20	0·25	0·10	0·15	0·15	$\bar{1}$·18	0·67
3	40	0·20	0·10	0·10	0·10	$\bar{1}$·00	$\bar{1}$·89
4	80	0·15	0·10	0·05	[illegible]	$\bar{2}$·48	$\bar{1}$·37
5	160	0·13	0·10	0·05	0·05	$\bar{2}$·48	$\bar{1}$·37
6	320	0·15	0·10	0·05	0·05	$\bar{2}$·48	$\bar{1}$·37
7	640	0·13	0·13	0		∞	
8	1280	0·13	0·13	0		∞	
9	2560	0·13	0·13	0		∞	
10	$\bar{x}$ =	0·13	0·13	0		∞	

Table 7

5. Antigen Plate - 1:384 dilution

	1	2	3	4	5	6	7	8	9	10
10						×				
20						×				
40							×			
80							×			
160							×			
320							×			
640							×			
1280							×			
2560							×			
C							×			

	x	z	u	z−u	f	log f	w
1	10	0·20	0·13	0·07	0·07	$\bar{2}$·85	$\bar{1}$·74
2	20	0·20	0·10	0·10	0·10	$\bar{1}$·00	$\bar{1}$·89
3	40	0·16	0·10	0·06	0·06	$\bar{2}$·78	$\bar{1}$·67
4	80	0·13	0·10	0·03	0·03	$\bar{2}$·48	$\bar{1}$·37
5	160	0·13	0·10	0·03	0·03	$\bar{2}$·48	$\bar{1}$·37
6	320	0·13	0·10	0·03	0·03	$\bar{2}$·48	$\bar{1}$·37
7	640	0·13	0·13	0		∞	
8	1280	0·13	0·13	0		∞	
9	2560	0·13	0·13	0		∞	
10	v =	0·13	0·13	0		∞	

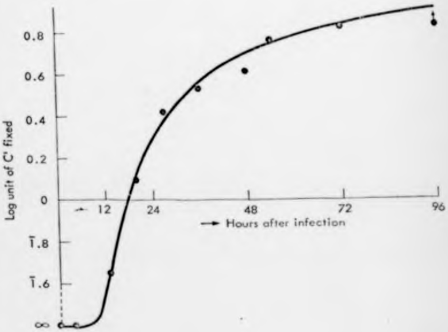
Table 8 . COMPLEMENT DILUTIONS

Column	Dilution (1:)	Amount of c' (µl) in 25 µl of dilution	Geometric Mean (µl)
1	12.6	1.99	
			1.57
2	20	1.25	
			0.99
3	32	0.78	
			0.62
4	50	0.50	
			0.39
5	80	0.31	
			0.25
6	126	0.20	
			0.16
7	200	0.13	
			0.10
8	316	0.08	
			0.06
9	500	0.05	
10	[illegible]	0	

Reovirus type 1 (Lang strain) after cultivation at 36°C was assayed by complement-fixation test. In Fig. 16 it is observed that the antigen contours indicate that the antigen concentration determines the maximal amount of complement which can be fixedat any antibody concentration. Hence, the maximal units of complement fixed are independent of the antibody concentration over the range of antiserum titre. It is therefore feasible in the assay to choose one level of antiserum dilution in which the antibodies are in great excess. Under these conditions, no pro-zone effect would be shown by the most concentrated tissue culture harvest. It was for these reasons that cell-free extracts of infected cultures were titrated undiluted against 1:10 dilution of antiserum.

In Fig. 18 it is shown that an exponential increase of complement-fixing antigen was observed after 9 hours. Four days after infection when the experiment was terminated, the amount of antigenic material produced by the cells fixed about 6.8 units of complement.

FIG 18. PRODUCTION OF COMPLEMENT-FIXING ANTIGEN OF REOVIRUS TYPE 1 (LANG STRAIN) AT 36°C IN ADULT RHESUS MONKEY CULTURES DERIVED FROM THE BRAIN AS ASSAYED BY COMPLEMENT-FIXATION TEST.



Infected Leighton tube cultures stained by the indirect fluorescent-antibody technique revealed specific granular, cytoplasmic inclusions at 15 hours after infection. Definite cytoplasmic aggregates were seen in about 15-20% of the cells at about 24 hours after infection. These aggregates were scattered in the cytoplasm and at 48 hours most of them had condensed into a large inclusion which displaced the nucleus. By the third day the inclusion had increased in size considerably and completely displaced the nucleus to the periphery of the cell (Fig. 19a, left).

In some large multipolar cells the granular inclusions increased in number to fill the whole cytoplasm. This was followed by the fusion of the granules into filaments or threads. Simultaneously a number of the granular inclusions aggregated to form a perinuclear ring (Fig. 19 a, right). Infected bi-polar (glial) cells showed a more advanced growth of virus by the third day. Figure 19 b shows a glial cell completely filled with a mass of fluorescent material. These various stages of inclusion formation were detected in about 50-60% of the cells. By the fifth day about 90% of the cells showed the presence of specific antigenic material in different inclusion forms.

No specific fluorescence was detected in the nucleus at any stage of viral growth and infected cells treated with normal rabbit serum followed by FITC-labelled immunoglobulin did not show any of the specific cytoplasmic fluorescence described above.

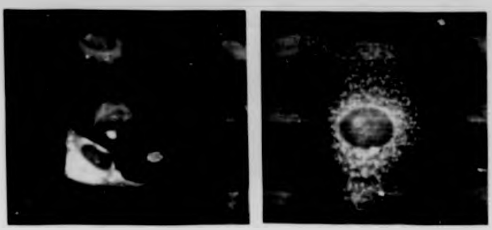


Fig. 19 a. Cultured adult rhesus monkey brain cells 3 days after infection with reovirus type 1 (Lang strain). Stained by the Sandwich antibody technique.

Left: Brilliant fluorescent inclusions surrounding the nucleus of triangular-shaped cell. x 640

Right: Granular inclusions in large multipolar cell. Note perinuclear aggregation; in areas distal to the nucleus fluorescent threads are visible in cytoplasm. x 640

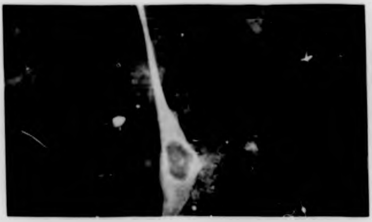


Fig. 19 b. Infected preparation as above showing the cytoplasm of a bi-polar (glial) cell occupied by fluorescent inclusion. x 640. The inclusions are yellowish instead of apple-green in the prints. This is due to over-exposure of the prints.

iii. Numerical example of estimating an antigen titre and its standard error. The antigen was Reovirus type 1 (prototype strain Lang) grown in cultured cells derived from the brain of adult rhesus monkey. Data from Tables 7a, b, and c.

	Antigen Concn. In 25 µl			Antiserum Concn In 25µl	
Dilution	x(µl)	log x	Dilution	y(µl)	log y
1.48	0.52	$\bar{1}.72$	1:10	2.5	0.40
1:96	0.26	$\bar{1}.42$	1:20	1.25	0.10
1:192	0.13	$\bar{1}.11$	1:40	0.625	$\bar{1}.80$
1:384	0.065	$\bar{2}.81$	1.80	0.3125	$\bar{1}.50$

$$w = \log_{10} (f/k)$$

antigen →	$\bar{1}.72$	$\bar{1}.42$	$\bar{1}.11$	$\bar{2}.81$
antiserum ↓				
0.40	0.58	0.46	0.15	$\bar{1}.74$
0.10	0.23	0.21	0.07	$\bar{1}.89$
$\bar{1}.80$	$\bar{1}.89$	$\bar{1}.89$	$\bar{1}.89$	$\bar{1}.67$
$\bar{1}.50$	$\bar{1}.67$			

The method of least squares is used for estimating the titre of the antigen as follows:

x	x'	$x'-\bar{x}'$	w	$w-\bar{w}$
$\bar{1}.72$	-0.28	+0.455	0.58	0.3475
$\bar{1}.42$	-0.58	+0.155	0.46	0.2275
$\bar{1}.11$	-0.89	-0.155	0.15	-0.0825
$\bar{2}.81$	-1.19	-0.455	$\bar{1}.74$ (-0.26)	-0.4925

$\Sigma x' = -2.94$ $\Sigma w = 0.93$

$\bar{x}' = -0.735$ $\bar{w} = 0.2325$

$$\Sigma (x' - \bar{x}')(w - \bar{w}) = 0.4303$$

$$\Sigma (x' - \bar{x}')^2 = 0.4620$$

$$b = \frac{\Sigma (x' - \bar{x}')(w - \bar{w})}{\Sigma (x' - \bar{x}')^2} = 0.9313$$

Using the equation,

$$w = \bar{w} + \beta (x - \bar{x})$$

and substituting β for b, we have

at $w = o$ i.e. I unit of complement

$$o = 0.2325 + 0.9313 (x_t + 0.735)$$

$$x_t = -0.9840$$

$$= \bar{1}.0160$$

antilog x_t = X_t = 0.1038μl of antigen in 25μl

Estimated antigen titre = 1:241

Calculation of standard error

	← z values →				
Antigen dilutions →	48	96	192	384	C
Antiserum dilutions ↓					
10	0.62*	0.50	0.31	0.20	0.13
20	0.31	0.31	0.25*	0.20	0.10*
40	0.20	0.20	0.20	0.16*	0.10*
80	0.16*	0.13	0.13	0.13	0.10*
C V	0.13	0.13	0.13	0.13	0.13=k

* Estimates of z denoted by the geometric mean of two levels of complement.

$\theta = 18/25 = 0.72 \quad 1 - \theta = 0.28$

where θ is the proportion of estimates of z in the experiment which are denoted by partial lysis and

$(1 - \theta)$ is the proportion of estimates of z in the same experiment which are denoted by the geometric mean of two levels of complement.

The standard error of the estimated titre [illegible] is obtained from the f values not from w = log f/k values.

[illegible]

Antigen (µl) →	0.52	0.26	0.13	0.0625
Antiserum (µl) ↓				
2.5	0.49	0.37	0.18	0.07
1.25	0.21	0.21	0.15	0.10
0.625	0.10	0.10	0.10	0.06
0.3125	0.06	0.03	0.03	0.03

The largest values of f at each of the four antigens are used. The best fitting straight line to these values indicates that at antigen concentration 0.52µl a maximum has not necessarily been reached. Therefore, the point ($f = 0.49$, $x = 0.52$) is ignored.

In the data for z values above, we have

$$u = v = k = 0.13;\ h = 0.2$$

$$(f_1, x_1) = (0.37, 0.26)$$

and $$(f_2, x_2) = (0.07, 0.065)$$

$$F = f_1 - f_2 = 0.30$$

Since neither antiserum nor antigen show pro- or anti-complementary effects ($u = k = v$), the standard error is estimated using condition (4) to define S_1, S_2 and S_3. In the calculation of the sums S_1, S_2 and S_3, θ or $(1 - \theta)$ is used depending on the type of z_1, z_2 and x.

$S_1 = z_1^2\ (\sigma)^2 = (0.50 \times 0.72)^2 = 0.1296$

$S_2 = z_2^2\ (\sigma)^2 = (0.20 \times 0.72)^2 = 0.02074$

$S_3 = 4t^2(\sigma)^2 = 4\ (0.13 \times 0.72)^2 = 0.0350$

$$S_x^2 = \frac{0.442h^2 \left[(X_2 - X_t)^2 S_1 + (X_1 - X_t)^2 S_2 + (X_2 - X_1)^2 S_3\right]}{F^2}$$

$$= 0.0003994$$

95% confidence limits for X_t are

$$X_t \pm 2S_x = 0.1038 \pm 0.03917$$

$$= 0.0646\mu 1 - 0.1430\mu 1$$

(C) COXSACKIE VIRUSES (A7 and B3)

The existence of the Coxsackie group of viruses in the picornaviruses family was first reported by Dalldorf and Sickles (1948). Extracts of faeces of two boys suffering from paralytic poliomyelitis provided the source from which the first strains of Coxsackie viruses were isolated. These two isolates were classified later as members of Group A Coxsackie viruses of which there are now twenty-three subtypes. Other types, subsequently to be known as the Group B viruses, were isolated from patients with non-paralytic poliomyelitis or aseptic meningitis (Melnick *et al.*, 1949) and from the faeces of "poliomyelitis" patients another virus which was pathogenic for suckling mice and differed from poliomyelitis virus but its host range and affinity for striated muscle was recovered (Dalldorf *et al.*, 1949). The Group B comprises six subtypes. The involvement of some Group A viruses in herpangina was shown by Huebner et al., (1951) and epidermic myocarditis in newborn infants has been demonstrated to be caused by Coxsackie B virus infection (Gear, 1955). A Group B Coxsackie virus type 2 was isolated from the heart muscle of a baby boy who died after a feverish attack with signs of myocarditis (Gear, 1958).

Both Group A and B viruses are small spheres and have cuboidal symmetry with approximately 42 capsomeres. Electron microscopic studies of purified viruses harvested from infected mice or tissue cultures yielded values of 28nm as the size of the particles (Breese and Briefs, 1953; Mattern, 1962). Purification by dialysis and ultracentrifugation of tissue of suckling mice infected with type A10 yields dodecahedral crystals with four hexagonal faces. The maximum dimensions of the crystals were found by Mattern and du Buy (1956) to be about 100nm and from crystalline preparations of Coxsackie virus A9 and A10, values of about 27-30nm were obtained for the diameter of the infective particle (Mattern, 1962). Molecular weight determination by Mattern (1962) of the A10 virus, having hydrated density of 1.34 g/cm^3 and hydrated diameter of 28nm and water of hydration assumed to be 30%, gave a value of 7 x 10^6 daltons.

The Coxsackie virus particle contains RNA in its core and infectious RNA has been extracted from both Group A and B viruses (Mattern, 1962). The RNA content has been demonstrated by the lack of inhibition of replication of Coxsackie A21 (Coe virus) by 5-iododeoxyuridine (IUDR) (Abraham, 1962). Growth of Coxsackie viruses in tissue culture is inhibited by guanidine (Rightsel *et al.*, 1961) but the inhibitory action of 2-(--hydroxybenzyl)-benzimidazole (HBB) is effective on intracellular growth of only A9, A21 (Coe virus) and the Group B viruses but not on types 7, 11, 13, 16 and 18 of Group A viruses. It has been suggested that both guanidine and HBB inhibit the production of RNA polymerase (Baltimore *et al.*, 1963) and of viral RNA and coat protein (Eggers and Tamm, 1962; Crowther and Melnick, 1961). Eggers and Tamm (1963b) have shown that when guanidine and HBB were used at their non-effective concentrations in a mixture, a synergistic action was effected by inhibiting the growth of either A9 or B3 in cell culture.

The Coxsackie viruses are stable at room temperature between pH 2.3 and 9.4 for 1 day and for 7 days between pH 4.0 and 8. They are more resistant in hydrochloric acid solutions than in veronal acetate buffers of the same pH values (Robinson, 1950). They are inactivated at 60°C in 30 minutes. In the presence of $1M\text{-}Mg^{++}$ and $1M\text{-}Ca^{++}$ Coxsackie viruses are stable at 50°C for 1-3 hours (Wallis and Melnick, 1962) but the use of $2M\text{-}Na^{+}$ markedly reduces their stability after one hour. When Coxsackie A21 virus was heated at 50°C, a 99.99% reduction in the infectivity resulted within 4 minutes. At 37°C, there was complete survival of virus for 24 hours, 10% survival after three days, 1% after five days, and 0.01% survival after 20 days. The infectivity titre decreased by 1.5 logs after 20 days at 25°C and 4°C (Parson *et al.*, 1960).

The Coxsackie viruses are resistant to 70% ethanol, 5% lysol and ether but they are inactivated rapidly by 0.1N HCl or 0.3% formaldehyde (Melnick, 1951).

Coxsackie viruses haemagglutinate red blood cells of different species. Type A7 virus agglutinate chick red cells which are positive for vaccinia (Grist, 1962); types A20, A21 (Coe virus), A24 agglutinate human group O erythrocytes (Johnson *et al.*, 1961; Rosen and Kern, 1961) and types B1, B3, B5 have been reported by Rosen and Kern (1961) to agglutinate human group O (new-born) erythrocytes. Studies by Schmidt *et al.*, (1963) on the haemagglutination of Coe virus have shown that treatment of the virus with fluorocarbon resulted in the induction or increase in titre of haemagglutinin for human group O erythrocytes. Two viral particles have been recognised with respect to haemagglutination. The HA-positive particles, initially dominant in most natural specimens, were selectively propagated by passage in primary human tissue cultures and high HA titres were obtained after consecutive passages at low dilution. The HA-negative particles, initially present in small numbers, are selected by passage in aneuploid cell cultures and after about five low dilution passages, HA activity is completely lost. The HA activity cannot be restored by growth in human embryonic kidney (HEK) cell culture after the activity has been lost (Johnson *et al.*, 1961; Johnson and Lang, 1962). The Coe strain does not agglutinate erythrocytes from man, fowl, guinea pig, rat, monkey, or sheep at 4°C or at 37°C without treatment (Fukumi *et al.*, 1958). However, fluorocarbon treatment at a pH of 5.8-6.8 yielded increase HA titre of HA-positive strains and haemagglutination by other strains was induced. Schmidt *et al.*, (1963) have suggested that the haemagglutinin is probably associated with the infectious particle. The Coe virus haemagglutinin is inactivated at 37°C, but at 4°C it remains stable for 24 hours.

The Coxsackie viruses have been propagated in a number of cell cultures. Among the group A viruses, type A9 grows readily in MK cells and most of them, with the exception of types 1, 4, 5, 6, 19 and 22, have been adapted to grow in human amnion cells. Primary isolation of type A viruses (A11, 13, 15, 18 and 20) may be achieved in human cell cultures and A21 grows best in HeLa, Hep-2, KB and primary human embryonic kidney cell cultures. Studies with type A21 have shown that on primary isolation, growth of the virus leading to degeneration of inoculated cultures occurs between the 9th and 16th day of inoculation. The cytopathic effect in

HeLa cells consists of rounding off the cells with or without enlargement or shrinkage. The cytoplasm becomes granular with an ill-defined nucleus followed by cell fragmentation and separation of cells from the glass surface (Lennette et al., 1958). Cytological studies on infected cells stained with haematoxylin and eosin showed nuclear coarsening and margination of the chromatin with nuclear pyknosis (Bloom et al., 1962). Unlike types A2 and A4 which have been propagated successfully in chick embryo cell cultures, these cultures as well as monkey kidney and hamster kidney have been found refractory to type A21 infection (Lennette et al., 1958; Sickles et al., 1959).

EXPERIMENTAL

The prototype strain AB4 of Coxsackie virus type A7 and Coxsackie virus type B3 (prototype strain Nancy) were supplied by the Central Public Health Laboratory, Colindale, London. Both viruses were serially passaged in cultured cells derived from the brain of rhesus monkey at 36°C. Stock suspension of each virus was prepared in the same manner as described under the preceding sections.

Infectivity titration and serum neutralisation tests of stock suspension of Coxsackie virus type A7 were done in test-tube cultures of nerve tissue cells at 36°C. The stock suspension of Coxsackie virus type B3 was titrated in 1 to 3 day-old suckling mice which were inoculated intracerebrally with 0.01ml of serial dilution of the virus suspension. A cross-reaction test between the two virus suspensions using homologous and heterologous neutralising antisera were carried out by complement-fixation test.

RESULTS

The growth of Coxsackie A7 and Coxsackie B3 viruses in the cultures of monkey cells derived from the brain was slow even with a multiplicity of infection of 5:1. Cytopathic changes in the cells were not detected until about 30 hours after infection. The growth of these viruses produced cytopathic effects characteristic of the picornaviruses group. This consisted of rounding of cells which eventually came off the substrate. The onset of the cytopathic changes was markedly selective in the choroid epithelial cells. By the fifth day after infection all the epithelial cells had degenerated. Three days following inoculation when foci of CPE were evident, the medium in a set of infected cultures was changed every two days. The bi-polar cells, the spindle-shaped and multipolar cells were not all affected, as indicated by cytopathological changes, until about 12 days after infection. No inclusion bodies were observed in these cells. The late appearance of cytopathic changes in these cells might indicate either a slow cycle of replication of the viruses in them or it is possible that the cells supported virus growth after they had been repeatedly exposed to virus particles released from infected choroid epithelial cells. Such a situation would parallel a cell co-cultivation system in which one cell type is more sensitive to virus infection than the other.

Infectivity titration of the stock suspension of Coxsackie virus type A7 gave an estimated titre of 5.6 x 10^6 $TCID_{50}$/ml and $10^3 TCID_{50}$'s/ml of the virus were neutralised with 1:200 dilution of rabbit neutralising serum.

Serological Relationship between Coxsackie A7 and Coxsackie B3 viruses by Complement-Fixation Test

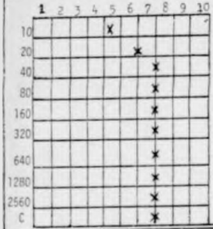
Suspensions of Coxsackie type A7 and type B3 viruses were compared in cross-reaction experiments by complement-fixation test using rabbit neutralising antisera. Attempts were made to standardise the antigens in terms of the amount of complement fixed in the presence of excess of homologous antibodies. The standardisation could not, however, be effected because the Coxsackie A7 antiserum was strongly anticomplementary at 1:10 dilution. The comparison was, therefore, conducted with dilutions of the antigens which gave most fixation of complement when each antigen was tested with its homologous antiserum.

For the comparison tests, nine 2-fold serial dilutions of each immune serum were prepared from 1:20 - 1:5120. A dilution of 1:40 of Coxsackie B3 antigen and 1:25 dilution of Coxsackie A7 antigen were titrated against homologous and heterologous antisera. A two-dimensional test was set up in each test with the antigen dilution as a plate constant, antiserum dilutions as row variable and complement dilutions as column variable. The mixtures consisted of equal volumes of 25µl of each component. After allowing the mixtures to react at +4°C overnight, 50µl of sensitised sheep erythrocytes was added to each mixture and incubated at 37°C for 2 hours.

Table 9

Titration of 1/25 dilution of Coxsackie A7 virus suspension against homologous antiserum.

1 Serum Control Plate - Diluent



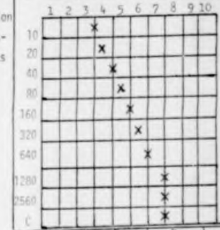
	x	U					
1	10	0.31					
2	20	0.16					
3	40	0.10					
4	80	0.10					
5	160	0.10					
6	320	0.10					
7	640	0.10					
8	1280	0.10					
9	2560	0.10					
10	$\bar{V}$ =	0.10					

k = 0.10

log k = $\bar{1}.0$

w = log f - log k

2 Antigen Plate - 1/2.5 dilution



	x	z	u	z−u	f	log f	w
1	10	0.63	0.31	0.32	0.32	$\bar{1}.51$	0.51
2	20	0.50	0.16	0.34	0.34	$\bar{1}.53$	0.53
3	40	0.40	0.10	0.30	0.30	$\bar{1}.48$	0.48
4	80	0.31	0.10	0.21	0.21	$\bar{1}.32$	0.32
5	160	0.25	0.10	0.15	0.15	$\bar{1}.18$	0.18
6	320	0.20	0.10	0.10	0.10	$\bar{1}.00$	0
7	640	0.16	0.10	0.06	0.06	$\bar{2}.78$	$\bar{1}.78$
8	1280	0.10	0.10	0	0	∞	—
9	2560	0.10	0.10	0	0	∞	—
10	V =	0.10	0.10	0	0	∞	—

Table 10

Titration of 1:40 dilution of Coxsackie B3 virus suspension against homologous antiserum.

1. Serum Control Plate - Diluent

	1	2	3	4	5	6	7	8	9	10
20								X		
40								X		
80								X		
160								X		
320										
640										
1280										
2560										
5120										
C							X			

	x	U						
1	20	0.09						
2	40	0.09						
3	80	0.08						
4	160	0.08						
5	320	0.10						
6	640	0.10						
7	1280	0.10						
8	2560	0.10						
9	5120	0.10						
10	X =	0.13						

k = 0.13

log k = $\bar{1}.11$

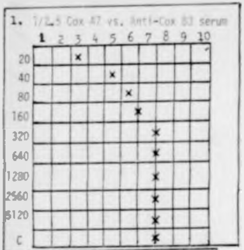
w = log f - log k

2. Antigen Plate = 1/40 dilution

	1	2	3	4	5	6	7	8	9	10
20										
40				X						
80						X				
160										
320										
640										
1280										
2560										
5120										
C							X			

	x	[illegible]	u	x−u	f	log f	w
1	20	0.99	0.08	0.91	0.91	$\bar{1}.96$	0.85
2	40	0.50	0.08	0.42	0.42	$\bar{1}.62$	0.52
3	80	0.20	0.08	0.12	0.12	$\bar{1}.08$	$\bar{1}.97$
4	160	0.10	0.08	0.02	0.02	$\bar{2}.30$	$\bar{1}.19$
5	320	0.10	0.10	0		∞	
6	640	0.10	0.10	0		∞	
7	1280	0.10	0.10	0		∞	
8	2560	0.10	0.10	0		∞	
9	5120	0.10	0.10	0		∞	
10	v =	0.13	0.13	0		∞	

1. 1/2.5 Cox A7 vs. Anti-Cox B3 serum



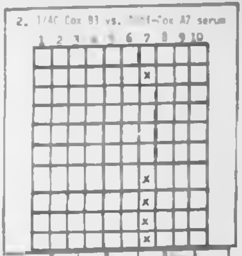
	x	z	u	z-u	f	log f	w
1	20	0·78	0·10	0·68	0·68	$\bar{1}$·83	0·83
2	40	0·31	0·10	0·21	0·21	$\bar{1}$·32	0·32
3	80	0·20	0·10	0·10	0·10	$\bar{1}$·00	0
4	160	0·16	0·10	0·06	0·06	$\bar{2}$·78	$\bar{1}$·78
5	320	0·10	0·10	0			
6	640	0·10	0·10	0			
7	1280	0·10	0·10	0			
8	2560	0·10	0·10	0			
9	5120	0·10	0·10	0			
10	$\bar{x}$ =	0·10	0·10	0			

Table 11

Serological relationship between Cox A7 and Cox B3

1. Cox A7 vs anti-Cox B3 serum
2. Cox B3 vs anti-Cox A7 serum

2. 1/40 Cox B3 vs. Anti-Cox A7 serum



	x	z	u	z-u	f	log f	w
1	20	0·16	[illegible]	0·03	0·03	$\bar{2}$·48	$\bar{1}$·37
2	40	0·15	0·10	0·03	[illegible]	$\bar{2}$·48	$\bar{1}$·57
3	80	0·10	0·10	0			
4	160	0·10	0·10	0			
5	320	0·10	0·10	0			
6	640	0·10	0·10	0			
7	1280	0·13	0·13	0			
8	2560	0·13	0·13	0			
9	5120	0·13	0·13	0			
10	$\bar{x}$ =	0·13	0·13	0			

Fig. 20

TITRATION OF COXSACKIE A7 AND COX B3 ANTIGENS AGAINST ANTI-COXSACKIE A7 SERUM.

DATA FROM TABLES 9 AND 11.

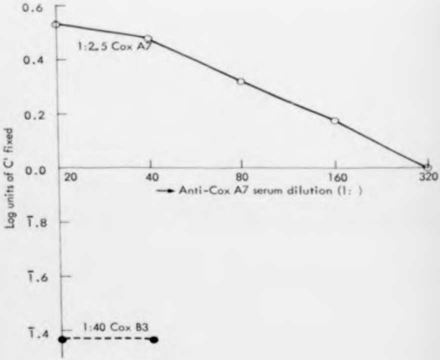


Fig. 21

TITRATION OF COXSACKIE A7 AND COXSACKIE B3 AGAINST ANTI-COXSACKIE B3 SERUM.

DATA FROM TABLES 10 AND 11.

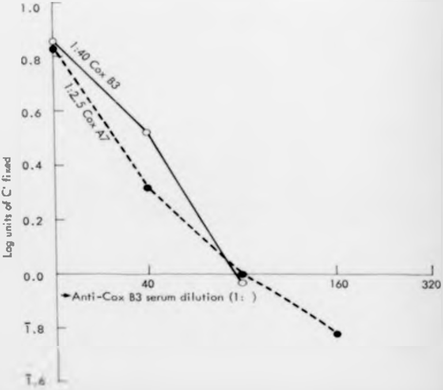


Fig. 22

TITRATION OF ORIGINAL SUSPENSIONS OF COXSACKIE B3 AND COXSACKIE A7 VIRUSES AGAINST ANTI-COX B3 SERUM. DATA FROM TABLE 12

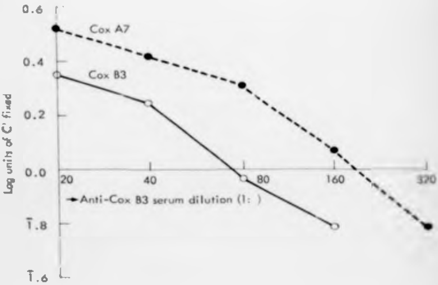
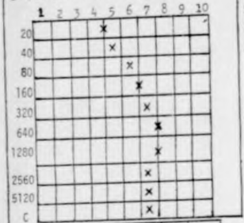


Table 12

Serological relationship between Coxsackie B3 and Coxsackie A7 antigens

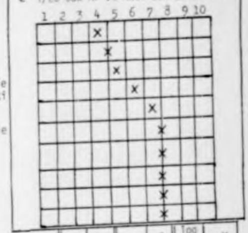
1. 1/25 dilution of Coxsackie B3 against homologous anti serum
2. 1/25 dilution of Coxsackie A7 against anti-Cox B3 serum

1 1/25 Cox B3 vs Anti-Cox B3 serum



	x	z	u	z-u	f	log f	w
1	20	0·39	0·10	0·29	0·29	$\bar{1}$·46	0·35
2	40	0·31	0·08	0·23	0·23	$\bar{1}$·36	0·25
3	80	0·20	0·08	0·12	0·12	$\bar{1}$·08	$\bar{1}$·97
4	160	0·16	0·08	0·08	0·08	$\bar{2}$·90	$\bar{1}$·79
5	320	0·13	0·08	0·05	0·05	$\bar{2}$·70	$\bar{1}$·59
6	640	0·10	0·08	0·02	0·02	$\bar{2}$·30	$\bar{1}$·19
7	1280	0·10	0·08	0·02	0·02	$\bar{2}$·30	$\bar{1}$·19
8	2560	0·13	0·13	0			
9	5120	0·13	0·13	0			
10	$\bar{x}$ =	0·13	0·13	0			

2 1/25 Cox A7 vs Anti-Cox B3 serum



	x	z	u	z-u	f	log f	w
1	20	0·50	0·10	0·40	0·43	$\bar{1}$·63	0·52
2	40	0·39	0·08	0·31	0·44	$\bar{1}$·53	0·42
3	80	0·31	0·08	0·23	0·26	$\bar{1}$·42	0·31
4	160	0·20	0·08	0·12	0·15	$\bar{1}$·18	0·07
5	320	0·13	0·08	0·05	0·08	$\bar{2}$·90	$\bar{1}$·79
6	640	0·10	0·08	0·02	0·05	$\bar{2}$·70	$\bar{1}$·59
7	1280	0·10	0·08	0·02	0·05	$\bar{2}$·70	$\bar{1}$·59
8	2560	0·10	0·13	-0·03	0		
9	5120	0·10	0·13	-0·03	0		
10	$\bar{x}$ =	0·10	0·13	-0·03	0		

The dilutions of complement at which 50% haemolysis occurred are recorded in Tables 9-11.The logarithms of complement fixed by the mixtures are also given. The logarithms of units fixed by each antigen were plotted against the serum dilutions.

The graphical representations of the results (Fig.20, 21). indicate that while Coxsackie A7 antigen cross-reacted with Coxsackie B3 antiserum, no cross-reaction was demonstrable between Coxsackie B3 antigen and antiserum to Coxsackie A7.

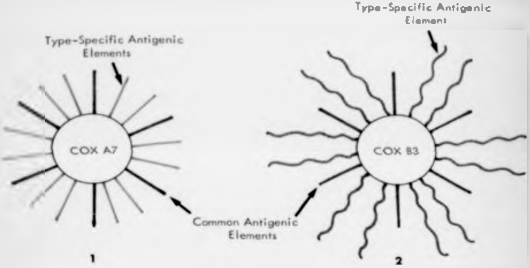
The test was repeated with the original suspensions of the viruses obtained from the Virus Reference Laboratory of the Central Public Health Laboratory in London. The cross-reaction between Coxsackie A7 antigen and Coxsackie B3 antibodies was confirmed (Fig. 22). The differences of the log units of complement fixed when the two antigens were titrated against the same set of dilutions of Coxsackie B3 antiserum and of the titres of antiserum at which 1 unit of complement was fixed by each antigen could be due to differences in concentrations of the two antigens used.

On the basis of the results indicating non-reciprocal cross-reactions, two types of hypothetical elements constituting antigenic structures for Coxsackie A7 and Coxsackie B3 viruses may be postulated. These are 1. type-specific antigenic elements and 2. specific antigenic elements common to both viruses. Fig.23 shows diagrammatic representations of the hypothetical structures for both viruses. The proportion of the common elements presented on each virus particle need not necessarily be the same as inidcated in the figure.

Consider the structural diagrams. On each virus particle there would reside both the common and type-specific antigenic elements. Antiserum raised to each of these would contain at least two types of antibodies. If this were the case, some cross-reactivity should have been detected when Coxsackie B3 antigen was titrated against antibodies to Coxsackie A7. In the absence of any, it is reasonable to consider the spatial distribution of the types of postulated elements on the two antigens.

Fig. 28

HYPOTHETICAL ANTIGENIC STRUCTURES FOR COX A7 AND COX B3 VIRUSES.



Two types of antigenic elements are proposed to reside on the two antigens namely common elements and type-specific elements.

1) Hypothetical antigenic structure for Cox A7 with both common and type-specific components in the same plane. This structure would provide sites equally accessible to specific IgG molecules for binding.

2) Hypothetical antigenic structure for Cox B3 with type-specific elements projecting beyond the antigenic elements common to both viruses. Such a spatial distribution would sterically hinder two specific IgG molecules occupying adjacent sites for Cl component of complement to bind.

Neutralising antiserum contains predominantly IgG antibody molecules. In the complement fixation reaction, two adjacent molecules of IgG are required for binding on to antigenic sites for the fixation of complement. In the case of IgM, a single molecule on a reactive site is able to bind C1 component of complement. A consideration of the diagrams would show that for the Coxsackie B3 antigen, two IgG antibody molecules to the common antigenic elements might not be able to bind on two neighbouring sites. This might result from steric hindrance due to the spatial distribution of the type-specific antigenic elements. If the configurations of the two antigenic elements were such as suggested for the Coxsackie A7 antigen, it would explain the easy accessibility of the antibodies to the common elements contained in Coxsackie B3 antiserum. Such binding would produce a reactive site, or sites, on Coxsackie A7 antigen for the fixation of complement to occur.

The spatial distribution of the two types of antigenic elements on Coxsackie B3 antigen could be tested for with early antiserum. Such antiserum should contain mostly IgM molecules which are the first type of antibody molecules to be produced in response to an infection or to an immunisation schedule. Each of these IgM molecules is required to bind on a reactive site to form a complex for complement to be fixed. It is suggested that the use of IgM molecules might, perhaps, circumvent the problem of non-reciprocity if any, and aid in establishing the existence of the postulated common antigenic elements, and the proportion of these elements residing on each antigen.

D. VACCINIA

Vaccinia, a member of the Poxvirus group, has been so extensively studied that only a brief summary can be attempted here. Electron microscopic studies of vaccinia-infected cells have revealed round or oval virus particles of variable size, density and inner structure (Gaylord and Melnick, 1953) and Morgan *et al.* (1954) observed that before virus particles are released from cells, many of them acquire a double membrane. The dense inner body or nucleoid has around it a less dense zone which is surrounded by a layer of denser material within an outer membrane which measures 9-12nm across. (Nagington and Horne, 1962; Noyes, 1962). Peters and Nasemann (1953) have estimated that the mature virus particles have the dimensions of 240-380nm x 170-270nm. Purified suspensions of vaccinia virus elementary bodies have been found to contain protein, DNA, phospholipid, neutral fat and carbohydrate (Smadel and Hoagland, 1942), but they do not contain alkaline phosphatase activity. The presence of enzymic activity reported earlier has been attributed to host-cell enzyme which the virus had adsorbed (Joklik, 1962). Studies on the DNA of purified suspensions have indicated that the nucleic acid is double-stranded (Müller and Peters, 1963) with a molecular weight of 80×10^6 (Joklik, 1962).

The virus can be kept indefinitely at -75° in the presence of peptone and the freeze-dried virus survives in nitrogen or *in vacuo* for many years. While dried virus withstands 100°C for 10 minutes, virus suspensions are inactivated in 10 minutes at 60°C. The pH stability of the virus of vaccinia ranges between pH 5 and 9 but at pH 3 it loses activity in 1 hour (Kaplan, 1958; Woodroofe, 1960; Sharp *et al.*, 1964). Wittman and Matheka (1958) have reported that the virus is resistant to ether extraction in the cold and to sodium desoxycholate but is inactivated by chloroform. It withstands the action of 1% phenol at 4°C for weeks but at 37°C may be inactivated in 24 hours. Potassium permanganate or ethylene oxide readily destroys the virus and it is inactivated by *p*-iodo-acetamide and other S-H reactive compounds.

Several distinct antigens are associated with tissues infected with vaccinia namely a nucleoprotein antigen (Craigie and Wishart, 1934; Smadel *et al.*, 1942), L-S antigen and haemagglutinin. The latter antigen, which is probably a phospholipid-protein complex, is separable from the virus particles by centrifugation and it withstands boiling. Chu (1948) estimated the diameter of the haemagglutinin to be 65nm. Datt (1964) demonstrated that the virus agglutinates red blood cells of turkeys, and some but not all fowl erythrocytes (Nagler, 1942). Spontaneous elution of virus from agglutinated cells does not occur (Burnet, 1946). The L-S antigen and nucleoprotein antigen can be used for complement-fixation tests and precipitation reactions. The L and S components of the LS antigen could be degraded independently; the LS antigen is distinct from the haemagglutinin. Zwartouw *et al.* (1965) detected 8 antigens, which can be extracted from purified virus, by immunodiffusion tests in agar.

The virus of vaccinia grows readily on the chorio-allantoic membrane of 7 to 13-day chick embryos and with suitable dilutions large opaque plaques can be produced on the membrane in 48 hours. Bedson and Dumbell (1961) have demonstrated that pocks can be formed at temperatures up to 40.5°. The virus can be grown in cultures derived from chick embryo, rabbit kidney and testis, bovine embryo and HeLa cells. The growth of the virus in cells produces cytopathogenic changes which include giant-cell formation and reticulum formation from lengthening of cytoplasmic processes as early as 48 hours. Intracytoplasmic inclusion bodies, basophilic and eosinophilic, occur. The mode of growth of vaccinia virus in cells has been studied by Cairns (1960) and Loh and Riggs (1961). One hour following the entry of virus particles into the cell by phagocytosis, vacuoles form within the cytoplasm. While still in the vacuoles, disruption of the coat of the particles occurs and no infective virus can be demonstrated from the host cell during this eclipse phase. DNA synthesis follows within the cell and in about three hours virus antigen is demonstrable with fluorescent anti-LS serum. The nucleoprotein antigen, which is synthesized independently from the LS antigen, appears a little later. Electron micrographs published by Dales and Siminovitch (1961) illustrate the viroplasm which may constitute the assembly of the LS antigen and the nucleoprotein together.

EXPERIMENTAL

A neurotropic strain of vaccinia (Levaditi strain), which had been passaged at least 50 times in developing chick embryos, was obtained from Dr. C.J.M. Rondle of this department. This stock virus was assayed by plaque counts. Serial ten-fold dilutions of the virus in PBS were inoculated on to the chorio-allantoic membranes of 7 day-old developing chick embryos. After 3 days at 36°C, the infected chorio-allantoic membranes were harvested into plastic petri dishes containing phosphate buffered saline. The membranes were washed and the number of plaque forming units per ml of the stock virus suspension was calculated from the plaque counts.

Secondary cultures derived from adult rhesus monkey brain cells were used for studying the mode of growth of the virus at 36°C. Leighton tube cultures which had been growing for 48 hours at 36°C were inoculated with stock virus diluted 10^{-3} in maintenance medium. One ml of the medium was delivered into each tube before they were re-incubated at 36°C. Specimens of infected and uninfected control cultures were removed at intervals, stained with acridine orange and examined with ultra-violet light.

Similar monolayer cultures grown in $25cm^2$ Falcon flasks were used for the one-step growth experiment of the virus in the cells. The same procedure used for growth curve studies described above was followed. Cultures were inoculated with diluted stock virus at an estimated input multiplicity of 3:1. After adsorption at 36°C for 1 hour, the cultures were washed with three changes (5ml each) of maintenance medium. Each flask received 2ml of maintenance medium and was then incubated at 36°C. Samples were removed at intervals during a 24 hour incubation period and frozen at -50°C. When all the samples were obtained, the infected cultures were harvested by freezing and thawing. After a light centrifugation to sediment cellular debris, the supernatants were assayed for total virus by complement-fixation test. In the test, two non-variable components were used - a strong concentration of inactivated rabbit anti-vaccinia serum (10^{-1} dilution) and undiluted, infected tissue culture harvests. These were titrated against varying dilutions of guinea-pig complement. Under these conditions, the maximal amount of complement that can be fixed is determined by the antigen concentration because at the optimum antiserum dilution, which was pre-determined by a three-dimensional complement fixation test, the antibodies are in excess of the complexing capacity of the most concentrated antigen used.

Using the plate technique, the squares in each row received 25µl of nine varying complement dilutions, an equal volume of 10^{-1} dilution of antiserum and an equal volume of undiluted tissue culture harvest under test. The tenth square in each row received a mixture of equal volumes of diluent, antiserum and the TC harvest under test. Two rows served as controls: one of these received equal volumes of antiserum, diluent and complement, the other control row received antiserum, complement and undiluted, uninfected tissue culture harvest. The mixtures were kept at +4°C overnight. 50µl of the indicator system was delivered to each test mixture and incubated at 37°C for 2 hours. The number of complement units fixed by each test antigen was calculated and the curve representing the growth of vaccinia (Levaditi strain) was drawn with the log (units of complement fixed) as ordinate against time (hours) as abscissa.

RESULTS

The growth of vaccinia (Levaditi strain) in cultured brain cells was associated with cytopathic changes which were discernible 4 hours after infection. Syncytia or giant cells were formed; the initial stages of the giant cell formation are depicted in Fig. 24 . A common observation was the lop-sided displacement of the nuclei of infected cells. As the cytopathic effect advanced, the cytoplasm of the infected cells retracted into round, crenated body which fluoresced pale orange. The initial stages of giant cell formation involved an infected cell enclosing within its cytoplasm a rounded-up cell. The syncytium progressed with the fusion of the cytoplasm of neighbouring infected cells with the primary syncytial growth.

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The haemadsorption (HA) test, used for detection of virus infected tissue culture cells (Shelokov et al., 1958) was performed on cultures which had been infected for 15 hours. Infected and uninfected Leighton tube cultures were removed and washed twice with maintenance medium. One ml of 2% suspension of washed turkey erythrocytes in maintenance medium was delivered into each tube. The cultures were incubated at 36°C to allow the erythrocytes to settle on to them. After 30 minutes the coverslips were washed with five changes of medium and stained with acridine orange. Fluorescence microscopy of the stained preparations showed that the HA phenomenon could be used to localise vaccinia infected cells. The nuclei of the erythrocytes fluoresced green: their stomata were not stained and therefore did not fluoresce. Fig. 25 shows the erythrocytes localised as clumps adherent to, and outlining a giant cell. Foci of haemadsorption were observed as rosettes in preparations which presented a number of individual, rounded-up cells. No haemadsorption was detected in uninfected cultures.

In order to determine the specificity of the HA reaction, infected and control cultures, treated with erythrocytes, were stained by the fluorescence antibody technique. The sandwich immunofluorescence technique of Weller and Coons (1954) was used. After adsorption on to the cultures, unadsorbed red cells were washed off and the cultures were fixed for 10 minutes in cold acetone. The coverslips were washed in distilled water, placed in plastic petri dishes and the monolayers were covered with rabbit anti-vaccinia serum diluted (10^{-1}) in Coon's buffered saline pH 7.2. After an hour's incubation at 36°C, the coverslips were washed in several changes of buffer which had been warmed to 37°C. They were covered with fluorescein isothiocyanate-labelled sheep anti-rabbit immunoglobulin (Wellcome, England) and incubated at 36°C for 1 hour. After washing in warmed Coon's buffer, the preparations were mounted in 90% glycerol in buffer.

Examination with the fluorescence microscope revealed bright apple-green fluorescent giant cells surrounded by turkey erythrocytes. The nuclei of the red blood cells were identified by the red fluorescence of their nuclei enclosed within oval shaped non-fluorescent stromata (Fig. 25).

Fig. 24 . Coverslip preparation of cultured adult rhesus monkey brain cells. Stained with acridine orange 4 hours after infection with vaccinia (Levaditi strain). Note syncytium showing one cell enclosed within another; also advanced CPE in cell in lower right hand corner. x 640

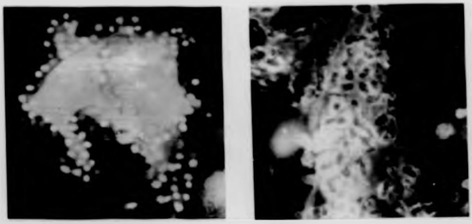


Fig. 25 . Cultures similar to one above, stained 15 hours after infection with vaccinia. x 640

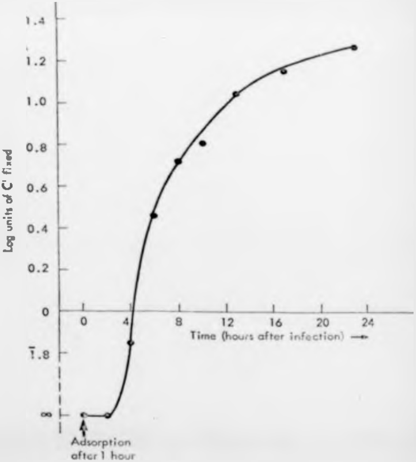
Left: A focus of haemadsorption - a giant cell with turkey cells adherent to it.

Right: HA followed by fluorescent antibody staining. Nuclei of turkey cells (orange) are adsorbed to fluorescent giant cell.

The production of complement fixing antigens by the Levaditi strain of vaccinia in cultured brain cells at 36°C, was assayed by complement-fixation test. A lag phase lasting less than 4 hours preceded the production of measurable amount of complement-fixing antigen by the fourth hour after infection. This coincided with the cytopathic changes observed microscopically. The rate of antigen production increased steadily up to 23 hours after infection when sufficient virus particles were formed to fix as much as 1.27 log units of complement (Fig. 26).

Phase-contrast microscopy of infected cultures revealed that the different cell types were equally susceptible to the cytopathic changes associated with the growth of the virus within 24 hours after infection.

FIG 26. PRODUCTION OF COMPLEMENT-FIXING ANTIGEN OF VACCINIA (LEVADITI STRAIN) AT 36°C AS ASSAYED BY COMPLEMENT-FIXATION TEST.



E. HERPES SIMPLEX VIRUS

The virus of herpes simplex (Herpesvirus hominis), Herpesvirus simiae (B virus) and Herpesvirus varicellae form a group in which each member is pathogenic for man.

Electron microscopy of virus particles negatively stained with phosphotungstic acid revealed that the virion consists of a core which measures 75nm. Surrounding the core is a capsid which measures 100nm in diameter and has a 5:3:2 axial symmetry of an icosahedron. The capsid of herpesvirus consists of 162 columnar capsomeres (9-10nm by 12-13.5nm) of which 150 hexamer capsomeres are distributed at the faces and edges and 12 pentamer capsomeres are located at the apexes of the icosahedron. An outer "envelope" surrounding the capsid is derived from host cell membrane and measures 145-200nm in diameter (Wildy *et al.*, 1960). Electron microscopic studies by Watson *et al.* (1963) showed that the particles may appear either "enveloped" or "naked". In either case, the particles may have their core intact or appear as empty particles without the core.

Holmes and Watson (1963) demonstrated with the electron microscope that both full "enveloped" and "naked" particles are infectious but adsorption of "enveloped" particles occur more readily than that of "naked" particles. After adsorption, the particles are ingested into pinocytotic vesicles in which they are carried toward the nucleus. During the transportation, "enveloped" particles are stripped of their covering by a digestive process and full "naked" particles are released in the paranuclear area, (Epstein *et al.*, 1964). It is believed that viral DNA then passes into the nucleoplasm where growth of virus occurs. The first sign of growth is the appearance of "primary bodies" 30-40nm in diameter which are seen in sections of infected tissues (Morgan *et al.*, 1959). Mature virus particles then appear in smooth-walled vesicles near the nucleus and eventually "leak" out of the cell. Epstein (1962) reported that leakage of virus particles from infected HeLa cells occurred by invagination of a vesicle wall or the cell wall membrane itself, thus acquiring a triple-layered envelope of host material.

Russell et al. (1963) have reported that the virus particles consist of 100 parts DNA, 25 parts carbohydrate and 320 parts phospho-lipid to 1000 parts protein. The viral DNA is double-stranded, has a density of 1.727gm/cm^3, a sedimentation coefficient of 44S and a molecular weight of 68 x 10^6 (Russell and Crawford, 1964). These workers also reported that the DNA has a guanine-cytosine content of [illegible].

The specific gravity of the particles is estimated at 1.27 - 1.29 and the isoelectric point at pH 7.2 - 7.6. Scott et al. (1961) found that several strains have a half-life of 90 minutes at 37°C and 3.75 hours at 30-31°C. At pH less than 6.8 and in the presence of divalent cations, the rate of heat inactivation is increased. However, the virus can be stabilised at 50°C by molar Na_2SO_4 and Na_2HPO_4 (Wallis and Melnick, 1965). UV-light and X-ray destroy the virus and various chemicals including ether, proteolytic enzymes and phosphatases are potent inactivators (Tokumaru and Scott, 1964).

Herpes simplex virus grows in developing chick embryo after inoculation by any route. On the chorio-allantoic membrane of 11 to 13-day old eggs, the virus produces conspicuous pocks between 36-48 hours after incubation at 36°C. The virus can be cultivated on several types of cells including rabbit kidney baby hamster kidney, chick embryo, HeLa and human amnion cultures. The cytopathic effect on tissue culture cells include inclusion body and giant cell formation. The virus of herpes simplex can be transmitted to guinea-pig, mouse, rabbit, hamster and cotton rat. Rabbits are very susceptible to infection via the cornea, skin, brain or testis. Neurotropic strains travel along nerves to the central nervous system from the cornea or other peripheral sites and cause convulsions and death from encephalitis.

The pathological changes in the nervous system occur mostly in the cortex with less involvement of the central white matter. Areas of the brain, especially of the temporal lobes, soften and frequently become haemorrhagic. The basal ganglia, the mid-brain, the brain stem, the cerebellum and the cord are relatively unaffected but the pons is consistently affected. Histologically, there is widespread mononuclear infiltration of the leptomeninges. The lesions in the cortex are characterised by intense degeneration and mild inflammation. This picture suggests encephalomalacia as a result of circulating disturbance. The choroid plexus is usually free of inflammatory changes. In areas of perivascular infiltration, degeneration of neurones is observed; and nuclear inclusions occur in the neurones and more frequently in the oligodendrocytes. Neurones are affected early, followed by glial cells. Fat-laden macrophages invade the field and neuronophagia occurs in necrotic areas. At the margins of necrotic areas, hypertrophied microglial cells and mild astrocytosis are seen by the 12th day. Demyelination occurs in the area of damaged cortex. In infants, the areas of necrosis tend to be more widespread and affect the basal ganglia and the brain stem (Haymaker et al., 1958).

The antigenic components of herpes simplex virus consist of neutralising antigens which are present in the viral particles and complement-fixing antigens which exist in both the viral particles and the soluble fraction. Four precipitating antigens have been detected by the double agar diffusion technique. These are the viral antigens which are separable by ultracentrifugation or trypsin and three soluble antigens which are separable in size, density and electric charges. Two of the soluble antigens are highly and one is slightly sensitive to tryptic digestion (Tokumaru, 1965).

Complement-fixing and neutralizing antibodies appear between the 4th and 6th days after primary infection and reach their peak by the 14th day.

Buddingh *et al.* (1951) reported that in children, the antibody may drop to undetectable levels after the primary infection but are boosted again by a series of subclinical or possibly clinical infections. The CF and neutralizing antibody levels are usually so stabilized by adulthood that they do not rise with the appearance of recurrent lesions.

EXPERIMENTAL

Herpes simplex virus type 1 (Strain 10711; 1970) was obtained from the Central Public Health Laboratory, Colindale, London. The virus was passaged two times in BHK cells. Stock virus suspension was prepared after cultures of brain cells derived from adult rhesus monkey had been demonstrated to support the growth of the virus. Three-day old secondary cultures of brain cells in 25cm^2 Falcon flasks were used for the preparation of stock virus suspension; the same procedure described for the preparation of stock Echovirus type 11 was followed.

Infected and control cultures for fluorescence microscopic studies were stained with acridine orange and by the indirect fluorescence-antibody technique.

Stock virus suspension was titrated in 3 to 4-day old suckling mice by quantal response. The mice were inoculated intracerebrally with 0.01ml of serial ten-fold dilutions of stock virus suspension.

RESULTS

Phase contrast microscopy of infected and uninfected confluent monolayers of secondary cultures revealed that the cytopathological changes observed in the cells resulted in the formation of large syncytial cells. The initial stages of the changes began with the appearance of micro-plaque-like foci of piling up of round cells. As the growth of virus progressed, the plaques extended in area to form giant syncytia. The cells eventually came off the substrate leaving patches of clear areas (macro-plaques) which could be counted under phase contrast microscopy. Because the formation of macro-plaques involved a number of cells cytopathic effects were not well established in the cultures until about 9 days after infection. After the first passage, semi-confluent monolayers of brain-cell cultures were used for subsequent passages. For the passages, confluent monolayers of cultures were subcultivated at a split ratio of 1:3 so that when the cultures were used by the 3rd to 4th day of incubation, most of the cells were not in direct contact with each other except the foci of epithelial cells. When these cultures were infected in parallel with confluent monolayers, it was observed that few giant cells were formed in semi-confluent monolayers. Furthermore the cytopathological changes in the cells were well advanced by the 24th hour after infection. Forty-eight hours following infection most of the cells in the infected cultures had rounded up. On the other hand, infected confluent monolayers exhibited the characteristic cytopathic changes leading up to syncytial cell formation and finally to patches of clear areas in the monolayers.

Infected and control cultures were stained with acridine orange and examined with the fluorescence microscope. Leighton tube cultures which had been infected for 24 hours showed marked cytopathic effect in the cells. The nucleus in rounded cells had retracted and condensed; the whole cell fluoresced pale orange to yellow (Fig. 27). Syncytia in similar cultures as above fluoresced brilliantly apple-green when infected cultures, stained by the indirect fluorescent-antibody technique, were examined under ultraviolet illumination (Fig. 28).

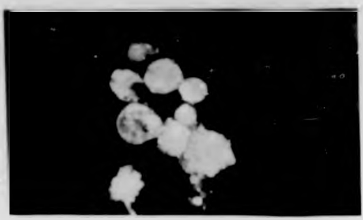


Fig. 27. Cytopathic effect in culture derived from adult rhesus monkey brain cells. Stained with acridine orange 24 hours after infection with Herpes simplex virus type 1 x 640

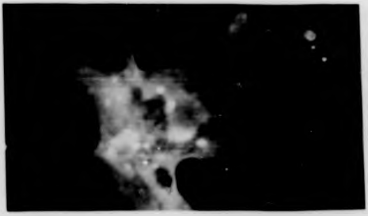


Fig. 28. Similar culture as above stained by the indirect fluorescent-antibody technique 24 hours after infection with Herpes Simplex Virus type 1. Apple-green fluorescence of a syncytial cell. x640

F. Tissue Affinity in Relation to Virulence of Coxsackie B3 Virus

Introduction

Emergence of variants of viruses with new characters which confer the property of virulence for a given host or tissue has been effected by empirical procedures. The new property, which may persist through several generations of viral growth, has been considered as a heritable feature. An example in point is the neurotropic strain of W-S strain of influenza virus. This variant appeared after successive serial passages of the virus by the intracerebral route in mice (Stuart-Harris, 1939). Neurovirulent strains of other viruses include vaccinia, yellow fever, herpes and measles. The appearance of such variants invariably involve the use of considerable number of animals. Furthermore, such *in vivo* adaptation does not lend itself to experimentation as to how the variants arise.

The proteins of the viral envelope, like those of the capsid, are determined by viral genes. However, the lipids and the carbohydrate moieties of glycoproteins are derived from the host cell, hence the virion surface may contain polysaccharide determined cellular antigens. Virus released from infected host cell may acquire a component derived from the host tissue; for example the typical icosahedral particles of herpes viruses are contained within an envelope which is derived from the cell membrane as the particles are released from the cell (Wildy *et al.*, 1960a). Russell *et al.* (1963) found that the envelope of herpes simplex virus contains 22% phospholipid. Analyses of viruses which contain lipids in their envelope structures have indicated that much of the viral lipid is derived directly from the cell membrane during release. For example, studies by Klenk and Choppin (1969b) have shown that when the paramyxovirus, SV5, was grown in cultures of monkey kidney and hamster kidney, the quantities of different types of SV5 lipids reflected in the lipid composition of the membranes of the two different cells. The data from the studies indicated that the same virus grown in different cell cultures can have varying composition of lipids.

The specificity of virus-coat protein in determining susceptibility to infection was demonstrated by Cords and Holland (1964). In this study, poliovirus nucleic acid was prepared with a coat of Coxsackie B1 virus and injected intraperitoneally into mice. It was found that mouse tissues which are normally susceptible to Coxsackie virus but resistant to poliovirus produced poliovirus.

The selection of virus particles from a mixed population may also govern the specific, target organ or tissue to which the virus is predisposed to infect. If the viruses which have been passaged in cultures derived from adult rhesus monkey brain have acquired some degree of specificity or affinity for this organ, as a result of selection, then the validity of the supposition could be tested. One of the viruses, Coxsackie virus B3 (Nancy), was used in this test. Coxsackie viruses grow in suckling mouse brain. Therefore, enhanced neurovirulence in this animal could be used as an index for determining differences in virulence between virus grown in cultures of monkey brain cells and in cultures derived from monkey kidney cells, possibly by a selection of virus from an heterogeneous mixture of viral population. The selected virus may therefore possess greater affinity for nervous tissue.

Experimental

The test virus had been passaged three times in cultures derived from adult rhesus monkey brain cells. On the fourth passage cultures were inoculated with virus at a low multiplicity of infection. It was designed that at an estimated multiplicity of 1:10, several cycles of infection would be initiated before complete cytopathic effect was established in the monolayers. The expectation was that through repeated *in situ* infections, successive generations of virus progeny would acquire adaptation to nervous tissue. When cytopathic effect was observed in about 90% of the cells, the virus was harvested by grinding the infected cells and subjecting the suspension to three cycles of freezing and thawing.

The test virus suspension and the original virus suspension were titrated in suckling mice by intracerebral inoculation of 0.01ml of ten-fold serial dilutions of each virus. The animals were observed for 9 days. The test virus had a titre of $8.79 \times 10^4 LD_{50}$/ml and the control virus a titre of $1.4 \times 10^4 LD_{50}$/ml. The control virus suspension was used at a dilution containing 100 LD_{50}/ml and the test virus suspension was diluted to contain an equivalent virus concentration per ml. 0.01ml of each inoculum was inoculated by the intracerebral route into 6 day-old suckling mice. Individual variations between litters were minimized by using the litter in each cage for both test and control virus suspnesions. The number of deaths was scored daily; the difference in the response of the mice in all the cages were statistically analysed by the X^2 test. Since the experiment was designed to compare the fatality rates between the two virus suspensions, it was deemed justifiable to use for the analysis the results obtained when a high mortality was observed in one set of mice.

Results

The results of the virulence test in suckling mice with Coxsackie virus B3 grown in two different cell strains are given in Table 13 below. The entries in the table are the ratios of the total number of deaths to the number of mice inoculated. The comparison between the fatality rates for the two virus suspensions was statistically determined by the X^2 test using a 2 x 2 contingency table of the total proportion of deaths on the 14th day following inoculation.

Table 13

Results of intracerebral inoculation of 6 day-old Suckling mice with Coxsackie virus B3 (Nancy) 14 days after inoculation

Cage	Test Virus	Control Virus
1	5/7	4/8
2	3/5	0/5
3	2/5	0/5

The comparison between the fatality rates for the two virus suspensions was statistically determined by the X^2-test using a 2 x 2 contingency table of the total proportion of deaths on the 14th day following inoculation.

Table 14

Fourfold table showing pooled results obtained on the 14th day after inoculation

Virus	Death	Survival	Total
Test	10	7	17
Control	4	14	18
Total	14	21	35

Table 15

Expected frequencies and contributions to X^2 for data in Table 14

Pooled results

Virus		Death	Survival	Total
Cox B3 (grown in monkey brain cultures	O	10	7	17
	E	6.8	10.2	17
	$(O-E)^2/E$	1.5059	1.0039	
Cox B3 (grown in monkey kidney cultures)	O	4	14	18
	E	7.2	10.8	18
	$(O-E)^2/E$	1.4222	0.9481	

The total contributions to X^2 from the 4 cells in the table above gives $X^2 = 1.5059 + 1.0039 + 1.4222 + 0.9481$
$= 4.8801$

From the X^2 tables, the observed value of 4.8801 is beyond the 0.05 point of the $X^2_{(1)}$ distribution and therefore the difference between the virulence of the two virus suspensions is significant at the 5% level.

These observations should be extended. Studies should be carried out on virus that has been serially passaged many times. Although there is, so far, no available information in the literature on the acquisition of neurovirulence by Coxsackie viruses after serial passage in mouse brain tissue, this too is an important aspect that should be pursued further.

6. DISCUSSION

In spite of the high rate of success which has been achieved in the cultivation of neurons of the peripheral and central nervous systems obtained from foetuses and neonates, the cultivation of the cells of adult nervous tissue has received much less attention and indeed less success. Costero and Pomerat (1951) found that the nerve cells from the cerebral cortex of the adult human brain were capable of undergoing profound morphological changes and there was no regeneration of the lost Nissl substance during the period of 4-5 weeks of maintaining the nerve cells _in vitro_. The degenerative and morphological changes found in most of the neurons in these cultures were attributed to the adaptation of the cells to the culture medium. Hogue (1953) and Geiger (1958) reported the survival and subcultivations of cells of adult human brain in long-term cultures, but the neuronal nature of the nerve cells in their cultures has been questioned (Murray, 1965). While Murray and Stout (1947) have successfully demonstrated the survival and multiplication of peripheral nerve cells from adult human beings, studies on the behaviour of the tissue of adult mammalian central nervous system _in vitro_ have been lacking.

The primary objective of this study was to investigate some aspects of the biology of adult nervous tissue _in vitro_ and for this purpose a simple tissue culture technique developed in this laboratory was adapted. One of the advantages of the method which was anticipated as ideally applicable to this tissue, was the retention of the fibrous matrix in which all the cellular elements of the brain are embedded. This advantage was considered a feature which supersedes the various culture techniques employed by neuroculturists in that no migration of cells occurred in the three-dimensional framework of the implanted tissue on polythene discs. The implanted fragments on the plasma-coated polythene discs were about 2 to 4 layers of cell thick and presented a model for studying the mode of spread of viral growth within this tissue.

The method of implanting tissue fragments on polythene discs facilitated the successful maintenance of motor neurons, pyramidal cells of the cerebral cortex and Purkinje cells of the cerebellum of adult rhesus monkey in vitro. The success would not have been attained but for media BA16-1 and BA16-2 which were formulated in the course of this study and in which neuronal and some glial elements have been maintained for 12 weeks and 14 days respectively.

The inability of the cells to support the growth of poliovirus type 1 (Mahoney) has been discussed already and ascribed to the inactivation or destruction of cell receptors for virus adsorption when the tissue was removed. It was found that neurons failed to survive in medium BA16-2 when the tissues were maintained in this medium from the outset. But if the tissue fragments were first adapted to medium BA16-1 (pH 3.0), neurons survived in medium BA16-2 (pH 6.5) for two weeks. Even under these conditions, infection of motor neurons could not be achieved. The explanation given above might not be far-fetched, for, since mature neurons do not grow, it is not unlikely to suppose that the receptors, without which virus infection could not be initiated, might not be capable of regeneration once they have been destroyed or inactivated.

The most intriguing observation made in the attempts to buffer medium BA16-1 with sodium bicarbonate was the pyknotic and chromatolytic effects of the buffered medium on the neurons. The cerebrospinal fluid contains about 25 mEq/l of HCO_3^- and the enzyme, carbonic anhydrase has been detected in neurons (Richter and Hullins, 1951). Since a search in the literature for any information on the utilisation of CO_2/HCO_3^- in the metabolism of differentiated neurons has been fruitless, a reasoned but contentious explanation is presented.

The detection of carbonic anhydrase in neurons would indicate that these cells can utilize CO_2 in a manner parallel to red blood cells.

Let us consider the following reactions:

Reaction 1: $NaHCO_3 + HCl \rightarrow NaCl + H_2O + CO_2$

" 2: $CO_2 + H_2O \rightleftharpoons H_2CO_3$

" 3: $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$

Since some amino acids in the medium are in the form of their hydrochloride derivatives, it is possible that addition of $NaHCO_3$ to the medium would generate CO_2 in situ according to reaction 1. The role of carbonic anhydrase in cells is to accelerate reaction 2 and by increasing the rate of formation of H_2CO_3, the formation of HCO_3^- would proceed more fully via reaction 3. The extent of the dissociation of H_2CO_3 would depend on hydrogen acceptors in the cell. The Nissl substances of neurones are RNA in nature and it is possible that as more H^+ ions are obtained from the dissociation of H_2CO_3, they would react with the NH_2 groups of the nucleic acid to form NH_3^+. This mopping up of H^+ ions would then continue until a steady state is reached in which the intracellular concentration of HCO_3^- relative to the extracellular concentration satisfied the conditions of the Donnan equilibrium.

On the other hand if the removal of the brain from the animal resulted in the inactivation of the enzyme, a slow build up of carbonic acid would result. But long before an appreciable amount of H_2CO_3 has been formed, the following reaction might also occur:

Reaction 4: $CO_2 + -NH_2 \rightarrow -NHCOOH$

If this reaction proceeded faster than reaction 2, the intra - cellular concentration of HCO_3^- would be greatly reduced. This would result in the displacement of the Donnan equilibrium which could be re-established by an influx of HCO_3^- from the extracellular fluid. But if the product of reaction 4 could not be metabolised and was present in a high amount, a condition parallel to acidosis might prevail intracellularly and lead to the pyknotic and chromatolytic changes observed in the neurons.

The morphological appearance of the cultures was normal for up to 84 days. Although degenerative changes occurred subsequently, evidence for the viability of such highly differentiated cells at low pH should be considered. The results of vital staining of the cultures with toluidine blue, neutral red and acridine orange supported the contention that the cells were viable as shown by the uptake of the dyes, whereas degenerated cultures were not stained. An attempt was made to determine whether the cells were actively metabolising by the use of radioactive purine and pyrimidine precursors (tritiated adenine and ^{14}C-labelled orotic acid). However, since several layers of cells were present in these preparations, the results of autoradiography were difficult to interpret due to background "noise".

The survival of neurons in medium BA16-1 might conceivably be due to the protection of the explant from the low pH by the plasma clot and the thickness of the tissue fragments. Cultures of primary monkey kidney cells and HeLa cells supported by a thin plasma clot, however, were destroyed in medium BA16-1. This observation provides additional support for the view that the neurons were viable.

Projected experiments include attempts to infect the neurons with naked poliovirus RNA and examination of the ultrastructural features of the cells by thin section electron microscopy.

Monolayer cultures derived from the cerebral tissue of adult rhesus monkey were successfully established in medium BA16-buffered with sodium bicarbonate and supplemented with 10% foetal calf serum. The cultures consisted of a mixed population of astrocytes, glial cells and choroid epithelial cells. These cells were consistently retained in serial subcultivation over a period of about 6 weeks when the cells finally degenerated. A speculative explanation for the degeneration has been presented in the relevant section. The important characterisitic of the cells was that under the conditions of growth, the cultures retained a normal karyotype configuration thus making them biologically similar to, or identical with, the same cell types in vivo. It is worth remarking that unless adult cells undergo transformation, they can divide only about twenty times in vitro. In this respect it is considered doubtful whether the long-term cultures of the cerebral cortex of adult human beings reported by Hogue and Geiger independently, were normal. Since karylogical studies were not carried out on the cells, it would be reasonable to suggest the possibility that the cells had dedifferentiated or transformed to have survived subcultivations for one to two years.

The monolayer cultures were shown to be capable of supporting the growth of some selected viruses which affect man. These were reovirus type 1, echovirus type 11, Coxsackie viruses types A7 and B3, vaccinia and herpes simplex virus type 1. The growth characteristics of the viruses in the cultures followed the pattern observed in various cell systems in which the viruses have been propagated but because a direct comparison could not be made during the course of this study, it is difficult to assess the sensitivity of the cultures derived from brain cells over those in current use for the isolation and propagation of the viruses. Interesting observations were, however, made on the susceptibility of the different cell-types in the cultures to virus infection. With the exception of vaccinia virus (Levaditi strain) which affected all cell types indiscriminately, when the cultures were inoculated under conditions in which all cell types could be infected equally and almost simultaneously, it was found that the choroid epithelial cells succumbed to infection, as judged by cytopathic changes in the cells, before the other cell types.

With the herpes simplex virus, it was also observed that giant-cell formation in confluent monolayers could be aborted when semi-confluent monolayers were used. The latter cultures were prepared by seeding the flasks with a low number of cells so that at the time the cultures were used, the individual cells were more or less in isolation from one another. The haemadsorption test was performed on vaccinia-infected cultures with turkey erythrocytes; the specificity of the reaction was confirmed by staining infected cultures by the fluorescent-antibody (Sandwich) technique after the haemadsorption test had been previously performed on the cultures. Driessen and Greenham (1959) used the haemadsorption test on vaccinia-infected HEK cells in an attempt to correlate haemadsorption centres with plaque counts. These workers concluded that even though the reaction was specific for vaccinia virus, it was more suitable for qualitative than for quantitative studies with the virus.

Another interesting result was observed in the studies with the Coxsackie viruses which were used. The determination of serological relationship between these members of groups A and B by complement-fixation test indicated that while Coxsackie A7 antigen cross-reacted with anti-Coxsackie B3 antigen and Coxsackie A7 antiserum. A hypothesis has been postulated to explain the non-reciprocity of the cross-reactions. The hypothesis was considered in terms of elements constituting the antigenic structures for the two viruses and the spatial configurations of the elements. A test has also been proposed to examine the validity of the hypothesis which if proven should form the basis for determining the complexity of antigenic structures not only among members in each group but more significantly between the two groups. Contreras et al. (1952) used complement-fixation and neutralization tests to classify Coxsackie viruses into antigenically distinct types. These workers found two-way cross-reactions between Conn-5 (B1) and Nancy (B3), Texas-14 and Nancy (B3), and Texas-12 and Easton-14 (A5), and indicated that Nancy has a broader antigenic constitution than the other Coxsackie viruses. They found also that there was a one-way crossing between Easton-10 serum and type-3 virus but there was no reaction between type-3 serum and Easton-10 (A8) antigen. It was also found that Texas-15 (A7) did not cross with any of the viruses and antisera tested. These two findings differ from what is reported in this thesis; the difference may be due to the fact that the strains of the viruses used by Contreras and associates were different from the prototype strains which were used for my studies. However, the differences emphasise yet again the need to know more about the antigenic structures of the viruses of the Coxsackie group.

Potential Application of the Cell Strain

A number of laboratories regularly use rhesus monkeys as a source of kidney for the preparation of primary cultures. The brain of these animals which are usually discarded could be used for the preparation of monolayers.

The search for viruses affecting the central nervous system still remains a protracted venture for which a tissue culture system derived from the brain of non-human primates should prove useful. The diploid monolayer cultures which have been successfully established in this project could be used for the study of viruses with neurotropic potential such as strains of measles virus for detailed investigation of their relationship, for example, with subacute sclerosing panencephalitis. In this context it is interesting to note that a neurotropic strain (Levaditi) of vaccinia and other non-neurotropic strains of viruses (e.g. herpes simplex virus 1 and ECHO 11) grew readily in the new cell-strain. Therefore adaptation to growth in nervous tissue culture may not be an *a priori* requirement for the application of the newly described cell-strain for virus studies.

Members of the alpha- and flavi-viruses of the Togavirus group (arboviruses) which cannot be isolated readily in currently available cell-strains and continuous cell-lines might grow readily in the cultures derived from adult rhesus brain cells. Of particular interest are the mosquito and tick-borne encephalitides. Preliminary studies have shown that Quaranfil, an unclassified member of the Togavirus group, and Lagos bat virus and Mount Elgon bat virus, both members of the rhabdovirus group, replicate in these cells whereas hitherto these viruses were isolated by intracerebral inoculation of mice and to a very limited extent in insect tissue cultures. However, further work is required in this direction.

It has been demonstrated that members of the Coxsackie A and B groups grow readily in the cell-strain but further experimentation is required to establish the application of this cell-strain for primary isolation of these viruses from clinical material. If successful, primary isolation would obviate the need to employ mice in diagnostic laboratories where animal house facilities are not always at hand. Cost effectiveness considerations are also of importance in this context.

The investigations of "slow viral infections" of the nervous system such as scrapie in sheep, mink encephalopathy, and Kuru and Creutzfeldt-Jakob disease in man, may be facilitated by tissue culture systems which could permit ready isolation and propagation. In this respect, the potential value of the cell-strain described here could be tested for. Recently, three viruses, J.C, SV40-PML and BK viruses, were isolated from brain tissues of cases of progressive multifocal leukoencephalopathy. These have been shown in serological studies to be readily distinguishable from one another antigenically, and all three share common antigens with SV40. The JC agent has been isolated in primary cultures of human foetal glial cells, and growth of the agent was demonstrated. The SV40-PML agent was isolated initially from cells cultured from a brain biopsy of a patient with PML. In this instance, virus was liberated when subcultures of the brain cells were fused with primary African green monkey kidney cells in the presence of inactivated Sendai virus. The three agents should be studied using the newly described non-human primate cell-strain derived from the brain tissue.

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APPENDIX

(1) Acetate-veronal buffer (MICHAELIS, L. 1931. Biochemie Zeitschrift, 234, 139).

Stock solution A

Sodium acetate ($3H_2O$)	9.714	gm
Sodium barbitone	14.714	gm
Glass-distilled water (CO_2 free)	500.0	ml

General formula for the preparation of working solutions of different pH values

Solution A	50	ml
8.5% NaCl solution	20	ml
N/10 HCl	X	ml
Glass distilled water (CO_2 free)	(180-X)	ml

Table for the buffering volumes (Xml) of HCl

X(ml)	pH	X(ml)	pH	X(ml)	pH
2.5	9.16	40.0	7.66	90	4.93
5	8.90	50	7.42	100	4.66
0	9.64	55	7.25	110	4.33
7.5	8.68	60	6.99	120	4.13
10.0	8.55	65	6.75	130	3.88
20.0	8.18	70	6.12	140	3.62
30.0	7.90	80	5.32	150	3.20
				160	2.62

(ii) Acridine orange solution

A 0.1% (w/v) solution of acridine orange (G.T. Gurr Ltd., London) was prepared in acetate-veronal buffer pH 7.6. The bottle containing the solution was kept in the dark.

(iii) Alsever's solution (modified) (BUKANTZ, REIN and KENT. 1946. Journal of Laboratory and Clinical Medicine. 31, 394).

Glucose	2.05
Sodium citrate	0.80 gm
Sodium chloride	0.42 gm
Glass distilled water	100.0 ml

The pH was adjusted to 6.1 by adding 1ml of 5% aqueous solution of citric acid to 100ml of solution. The solution was autoclaved at 15lbs for 15 minutes and stored at 4°C.

(iv) Azure eosinate stain (LILLIE. 1948. In Pathologic Technic. The Blakiston Company. Philadelphia and Toronto).

Stock solution

Azure eosinate stain	1 gm
Glycerol	50 ml
Methyl alcohol	50 ml

The stock solution could be kept indefinitely.

Working solution pH 4.27

Stock azure-eosinate solution	0.5 ml
0.1M citric acid in 25% MeOH in H_2O	1.2 ml
0.2M Na_2HPO_4 in 25% MeOH in H_2O	0.8 ml
Acetone	5.0 ml
Glass-distilled water to make up to	40.0 ml

The buffered stain was prepared each time it was required.

(v) Coon's buffered saline pK 7.2

Sodium barbitone	10.3gm
Sodium chloride	42.5gm
N/I hydrochloric acid	40.3ml
Glass distilled water to make up to	5 litres

(vi) Veronal buffer diluent for CFT (FULTON AND DUMBELL Journal of General Microbiology, 3, 97.

Stock solution

5,5-diethylbarbituric acid	5.75gm
Na-5,5-diethylbarbiturate	3.75gm
$MgCl_2$ $6H_2O$	1.68gm
$CaCl_2$	0.28gm
NaCl	85.00gm

The acid and salt were dissolved in 500ml of hot glass distilled water and the other components were added. The volume was made up to 2 litres and the solution was autoclaved at 15 lbs for 20 minutes. The diluent, which should have a pH of 7.2 was stored at 4°C. For use, the stock solution was diluted 1:5 in glass-distilled water.

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Reprinted from **CYTOBIOS**

Maintenance of adult rhesus monkey motor neurons in tissue culture

[illegible]

Abstract

[illegible]

Introduction

[illegible]

Materials and methods

[illegible]

[illegible] years of age when the [illegible] from Uttar Pradesh, India.

During their first week [illegible] at the National Institute the animals [illegible] were tuberculin-tested and clinically examined for monkey B virus. When any animal showed signs of illness, a course of antibiotics such as chloramphenicol, streptomycin or penicillin was administered depending on the symptoms. The above details were kindly supplied by Mr K. Hartley in a personal communication.

Maintenance medium

Media that have been shown by most investigators to support survival and growth of neural tissue, particularly of foetal and neonatal origin, proved to be detrimental to nervous tissue obtained from adult [illegible] monkeys and baboons. These media contain various combinations of foetal calf serum or horse serum and Eagle's MEM, or Simms's [illegible], bovine serum ultrafiltrate, human placental cord serum or human ascitic fluid and embryo extract.

After a fruitless search over a period of 1½ months for a medium in which to maintain adult nervous tissue, we decided to formulate a chemically defined, protein-free medium with the aim of mimicking the composition of cerebrospinal fluid.

The maintenance medium, which we have designated BA[illegible] and has proved successful, consists of a balanced salt solution, [illegible] Eagle's minimum essential amino acids, and [illegible] Eagle's vitamins. Details of the composition of the medium are shown in Tables 1 and 2.

The composition of the salt solution was based on the mean ionic concentrations of electrolytes found in human cerebrospinal fluid (Documenta Geigy, 7th edition). 'Analar' grade chemicals were obtained from British Drug Houses Chemicals Ltd. A single strength BSS was diluted from a concentrate [illegible] which was prepared in autoclaved glass-distilled water and sterilised by membrane filtration. Eagle's amino acids in concentrated form [illegible] and vitamins [illegible] were obtained from Biocult Laboratories.

The pH of medium BA[illegible] was between 6.8 and 7.0. This low value was due to the acidity of the commercial solution of amino acids, which include the hydrochloride derivatives of arginine, lysine and histidine. We have prepared and used a solution of amino acids with these three components as free base in a medium having a pH of 7.4, but without success. It was obvious then that HCl was needed in the medium.

Various buffering systems including CO_2/HCO_3 and zwitterionic compounds (MOPS, TRIS, HEPES, PIPES, BES) were also found unsatisfactory

Table 1 Composition of balanced salt solution

Salt	mg/litre
NaCl	6 [illegible]
KCl	224.0
$CaCl_2.2H_2O$	161.8
$MgSO_4.7H_2O$	270.0
$NaH_2PO_4.2H_2O$	90.66
Glucose	6 000

Table 2 Maintenance medium, BA16-1 [illegible]

Ingredient	Volume (ml)
Balanced salt solution (×20)	[illegible]
Glass-distilled water	[illegible]
Eagle's minimum essential amino acids (×50)	[illegible]
Glutamine (200 mM)	[illegible]
Eagle's vitamins (×100)	[illegible]
	[illegible]

[illegible]

[illegible] up to 6 days when most of the neurons were found to remain viable as judged by their morphological appearance after staining.

[illegible]

The animals were injected intramuscularly with 0.2 ml of sterile aqueous [illegible] the monkeys were drenched in a solution of 10% Chloros [illegible] ml of blood were obtained by cardiac puncture and the kidneys were removed aseptically for the preparation of primary cultures.

The back of the animal was thoroughly wiped with cotton wool to dry the fur and the skin removed with sterile instruments. The vertebral column was cut open and the spinal cord [illegible] the meninges and the dorsal root ganglia intact [illegible] median fissure with a sterile scalpel blade. The meninges [illegible] attached were separated, and each half of the cord [illegible]

Table 3 [illegible]

[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]
[illegible]	[illegible]

[illegible]

universal container full of medium BA16·1 for transportation. The procedure for other parts of the CNS was similar. The medulla oblongata was divided longitudinally into two, the vermis of the cerebellum and slices of the cerebral cortex were obtained by cutting sagittally with a sterile pair of scissors. Since most of the study was carried out with spinal cord tissue, detailed description of the culture technique will be directed to this tissue.

Preparation of cultures

The processing of the tissue began 2 hr after removal from the [illegible]. A piece of spinal cord was removed from the transport medium and placed on a sterile glass petri dish. The white matter was carefully pulled apart with a pair of sterile scalpel blades so that a strip of anterior horn grey matter with its underlying white matter was exposed. As much of the white matter as possible was dissected but no attempt was made to remove it completely. After dissection the tissue was moistened by immersing it briefly in the transport medium. Each strip of tissue was cut into about [illegible] measuring about 3 to 4 mm in length.

The tissue was implanted on polythene discs. The principles and details of the technique have been described previously by Fulton (1960). The tissue culture plates were placed in a desiccator and incubated at 36°C. The tap on the desiccator was left open so that there was a free gaseous exchange with the atmosphere. The medium was changed weekly either by pipetting off the medium and replacing it with fresh medium or transferring the cultures into another set of cups containing fresh medium.

Staining

In general, 4 different histological techniques employing the basic dyes Einarson's chrome alum gallocyanin, toluidine blue, cresyl fast violet and azure-eosinate were used to test for viability and identify the population of cells. Cresyl fast violet and azure-eosinate stains were found superior to the others and were used routinely to identify neurons by the presence of cytoplasmic basophilia.

Acetate buffered cresyl fast violet solution pH 4·5 (Manes, 1960) was used at a final concentration of 0·02%. The discs were stained at 60°C for 1 hr and then allowed to cool. They were washed briefly in distilled water, dehydrated through several changes of 95% alcohol until no more excess stain was removed and absolute alcohol. Clearing was done in two changes of 25% alcohol in xylene then xylene and mounted in DPX.

Lillie's azure-eosinate stain was used to detect necrotic changes in neurons. Discs were stained in buffered azure-eosinate (pH 4·1) at 60°C for 2 hr during which complete penetration of the stain into the deeper layers of the tissue took place. After staining the discs were rinsed briefly in distilled water and dehydrated with several changes of acetone. They were then cleared in two changes of acetone-xylene mixture (1:1) and two changes of xylene. The discs were mounted in DPX.

In all the staining methods tissue were fixed in 10% formol-saline for 30 min and sometimes for longer periods ranging up to about 10 hr. They were then washed in 4 changes of distilled water for 10 min before staining. More often than not the tissue separated from the discs during the clearing stages. This however, did not impair the mounting of the tissue, which was thick enough to be manipulated with a pair of forceps. The side of the tissue to which the stain had been applied was mounted uppermost on the slide. This was important for microscopic examination, since there was a gradient in the depth of staining of the different layers of cells.

Observations

Since Nissl substance contains a strongly acid protein, it was argued that survival and maintenance of fully differentiated mature neurons would be ensured if tissue of the CNS was maintained *in vitro* in a fairly acid medium. This assumption was partly borne out when preliminary experiments were carried out with Eagle's minimum essential medium without sodium bicarbonate (pH 4·0). Neurons retained their characteristic morphological appearance but the Nissl bodies remained granular and diffuse during the 3 weeks of observation.

Addition of small volumes of 7·5 % sodium bicarbonate solution to the medium to raise the pH to 7·3 was found to be detrimental to the survival of the nerve cells. After an overnight incubation period of 15 to 19 hr in the CO_2/HCO_3 buffered medium, nearly all the neurons in the explant had undergone complete chromatolysis. The nerve cells were observed as ghost-cells with grossly shrunken

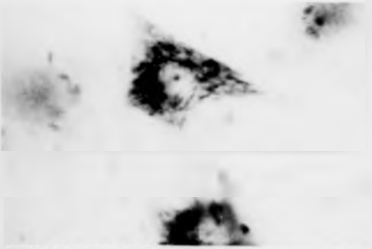

Figure 1a Motor neurons showing discrete Nissl substance in culture immediately after implantation. Figures 1a, 1b, 2 and 3 show nerve cells of the anterior horn grey matter of the lumbar enlargement of the spinal cord of [illegible]

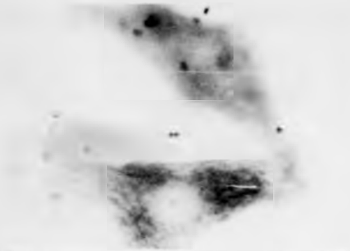

Figure [illegible]

nuclei. Cells that had not yet reached the last stages of degeneration stained very weakly with cresyl fast violet; their nuclei were filled with darkly stained granules, the product of nucleolar disintegration. Some of these granules were often seen as specks along the nuclear membrane.

The development of medium BAtb c was dictated by repeated failure to maintain neurons in various media which had been reported to support the growth of neurons in organotypic cultures of CNS obtained from embryos and very young animals. In media such as Eagle's MEM containing various concentrations of foetal calf serum or horse serum, or Simms's X7 containing bovine serum ultrafiltrate, human placental cord serum or human ascitic fluid and embryo extract, the chromatolytic changes were often severe. The number of healthy looking neurons in tissue fragments stained and examined immediately after implantation on to polythene discs was small and the staining reaction very weak. Their nuclei were variable in shape and eccentrically located in the perikarya. The Nissl bodies were dispersed and dust-like, and the short, rudimentary dendrites were barely visible. After overnight incubation neurons had disappeared. A few cells stained very weakly with cresyl fast violet; the cytoplasmic basophilia was finely granular and staining was much less intense than that observed in [illegible] preparations. With azure-eosinate stain cells were hardly distinguishable from the pink background.

Tissues transported and maintained in medium BAtb c, however, were morphologically characteristic of [illegible] neurons, showing cellular variation in shape (multipolar, pyramidal or triangular and elongated) and size (large, medium and small).

Immediately after implantation the nuclei of most of the neuronal population were either oval or round with a clear vesicular area. They were centrally located in the perikarya (Figure 1a), and each contained a round and darkly stained nucleolus within which could be seen an opaque body or small vacuole variously known, for example, as nucleololus (Dutta *et al.*, 1963).

In the cytoplasm of these cells the Nissl substance stained intensely, was flaky, discrete, and conspicuous in the dendrites, some of which measured about 270 µm in length. Axons were infrequently seen in our preparations; when these were thought to be present in freshly prepared cultures, they were identified by the absence of Nissl substance in the origin of the process (axon hillock).

In very large multipolar motor neurons and small cells with few or no processes, the chromidial material was granular but not diffuse (Figure 1b). These cells were not thought to be in stage 1 chromatolytic state because the nuclei and nucleoli were centrally placed or very nearly so and there was no viscous zone of acidophil cytoplasm adjacent to the nuclei. Cells were also present in some of the tissue fragments with centrally located nuclei and flaky Nissl substance. But the nucleoplasm had retracted into an oval shape around a darkly stained nucleolus and was bound by a clear area, the limits of which must have been the pre-existing, delineating nuclear membrane.

Most of these cellular features were faithfully and consistently present in specimens stained and examined weekly up to 84 days of incubation. The

Figure 2 [illegible]

[illegible] in ageing cultures which were observed included the progressive loss of Nissl substance by dendrites, their borders became less distinct, and the macroglial cells stained less intensely than younger cultures (Figure 2 and 3).

The effects on the nerve cells of buffering medium BA16-1 with CO_2/HCO_3 were reproducible and these have been described above. Zwitterionic compounds used as buffers produced within 24 h loss of cytoplasmic basophilia and shrinkage of the nuclei of neurons.

So far medium BA16-2 (Table 1) has been the only medium with a pH value higher than 7 [illegible] preparations. Media BA16-1 and BA16-3 have been found to function best after they were prepared and stored at room temperature for a minimum period of 5 days. [illegible] BA16-1 [illegible] BA16-2 [illegible] period and maintained in medium BA16-2 from the onset degenerated rapidly. However, if the tissue was initially maintained in medium BA16-1 [illegible] more the survival rate was greatly improved.

Discussion

Any culture technique that seeks to simulate the behaviour *in vivo* of central nervous tissue must enable the complex morphology of the cells *in vivo* to be successfully reproduced.

In organotypic and explant cultures of nervous tissue, cells like astrocytes migrate out of the explant denuding individual neurons *in situ*, thus permitting

Figure 4 [illegible]

microscopic examination. In the method described here, however, the cyto-architecture as well as the histology of the fragments is retained. The explanted fragments on the plasma-coated polythene discs are about 3 to 4 layers of cell thick, and present a three-dimensional network of cells in the deeper layers. No migration has been observed in any of our preparations including fragments derived from the cerebellum. One of the advantages of the technique is that the need for section cutting is obviated. It is therefore eminently suitable for rapid examination of cultures.

Administration of [illegible] the paralysis of the animal and [illegible] acts on motor neurons especially the neurons of cord grey matter. In addition, some trauma to the CNS tissue is inevitable during its removal. The successful recovery, survival and maintenance of neurons in our preparations are attributable to the medium BA16 1. Our working hypothesis for the development of this medium has been validated. It is, however, difficult to understand why adult nerve cells in vitro prefer an acid environment.

The use of medium BA16 2 will be vindicated when it can be shown to maintain nerve cells in a state suitable for the growth of neurotropic viruses. Then perhaps Murray's (1965) ultimate desideratum for a synthetic medium that will sustain advanced differentiation in complexes of nervous tissue will be realized.

Acknowledgements

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Hogue M. J. [illegible] **93** [illegible]

Kiernan J. A. and **Pettit D. R.** [illegible] **32** [illegible]

Marks F. [illegible] **35** [illegible]

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TISSUE CULTURE OF HUMAN EMBRYO LIVER CELLS AND THE CYTOTOXICITY OF AFLATOXIN B_1

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Attempts to grow differentiated human parenchymal liver cells in tissue culture have met with considerable difficulties (Bang and Warwick, 1965). Hillis and Bang (1962) maintained liver cells in culture for up to 100 days after primary explantation of liver fragments on reconstituted rat tail collagen in roller tubes. Festenstein (1963) maintained hepatic cells in culture for about 30 days by allowing suspensions of cells obtained from human embryo liver to settle by gravity on collagen-coated polythene discs. The main disadvantages of using methods involving primary explantation are those associated with the pluripotential nature of the complex of cells that make up the liver, and the problems presented by the types of cells that grow out from the implant and the necessity of attempting to select parenchymal cells by modifying the growth medium or the environmental conditions.

Attempts to obtain liver cell lines have also met with great difficulties. Chang (1954) described a cell line consisting of epithelial cells derived from normal human liver. The cells were grown in 20 per cent human serum and 5 per cent chick embryo juice, and subcultivated by trypsinisation. The cells developed the characteristics of undifferentiated neoplastic tissue and it is not suggested that these cells represent differentiated hepatic cells. In 1957, Syverton and McLaren obtained a cell line from a liver biopsy of an infant. The morphology of these cells was similar to the Chang cells.

To await the growth and multiplication of the complex of hepatic cells by these methods involves the chance of changes in morphology, differentiation and transformation of the cells. We therefore, attempted to use the method described by Fulton (1960) of direct implantation of fragments of solid organs on to polythene discs to form a monolayer of cells. This technique gives rapid separation of cells without destruction of the normal architecture of the tissue and without loss of viability. Furthermore, the implanted cells normally recover from the traumatic effects of the procedure within a few hours, and the monolayers so obtained can then be used (Arya and Fulton, 1965).

Our efforts to grow human liver in tissue culture were primarily aimed at an attempt to study the virus of acute hepatitis. We utilized these preparations in the meantime to observe the influence of aflatoxin on embryo liver cells (Zuckerman and Fulton, 1966).

The hepatotoxic effect of a number of metabolic compounds produced by fungi particularly the aflatoxins formed by strains of the *Aspergillus flavus-oryzae* group and islanditoxin and luteoskyrin present in *Penicillium islandicum*, has recently been recognised (Sargeant, Sheridan, O'Kelly and Carnaghan 1961; Lancaster, Jenkins and Philp 1961; Schoental 1961).

The aflatoxins consist of 4 distinct but chemically closely related substances known as B_1, B_2, G_1 and G_2, according to their fluorescence on chromatography under ultraviolet light. Observations have shown that the aflatoxins cause liver damage not only in poultry but also in lambs, pigs and calves; and in the laboratory, aflatoxins affected guinea-pigs, rats and young rhesus monkeys. Aflatoxin B_1 has been found to be the most toxic component but in addition to its acute hepatotoxic effects, aflatoxin B_1 has been shown to be a potent carcinogenic agent in rats, trout and ducks. There have been, however, very few studies on the activity of aflatoxin on cells grown in tissue culture. Smith (1963) briefly referred to vacuolation of monkey kidney cells growing in monolayers after aflatoxin B_1 was incorporated in the growth medium and to inhibition of growth and cell destruction with higher concentrations of the toxin. Juhasz and Greczi (1964) reported that extracts of groundnut meal samples contaminated with aflatoxin destroyed calf kidney cells in culture. Legator and Withrow (1964) noted that aflatoxin suppressed mitosis in human diploid and heteroploid embryo lung cells in tissue culture. More recently Legator, Zuffante and Harp (1965) using heteroploid embryo lung cells found that both crude aflatoxin and aflatoxin B_1 suppressed the synthesis of DNA and inhibited mitosis. Giant cell formation occurred and it was suggested that this could be accounted for by the enlargement of non-dividing cells. However, the effect of naturally occurring hepatotoxins on human liver cells has not been observed.

MATERIALS AND METHODS

Livers were obtained by the Tissue Bank of the Royal Marsden Hospital from human embryos, after abdominal hysterotomy, at stages of gestation varying from 8–22 weeks. The livers were removed under strict aseptic conditions, placed in Medium 199 and transferred to the laboratory on melting ice. The liver was either cultured immediately or kept overnight at 4°.

The apparatus used consisting of tissue culture trays and covers, polythene discs and Perspex plates, and the precise method of tissue implantation have been described in detail elsewhere (Fulton, 1966). Clotted mouse plasma was used as an adhesive. Certain modifications in the method of cell disaggregation and in the growth medium were necessary for the culture of human liver. Another critical factor in this technique which we attempted to measure was the total pressure exerted on the cells necessary to ensure viable implantation.

Methods of cell dispersion

Mechanical disaggregation. A portion of liver measuring about 1·5 × 1·5 cm, was placed in a test tube containing 5 ml. of modified Alsever's solution (Bukantz, Rein and Kent, 1946). The solution was aspirated up and down using a 1 mm. bore pipette until a fine suspension of cells was obtained. The suspension was passed through 2 layers of sterile gauze and centrifuged at 500 rpm for 5 min. The packed cells were washed in tissue culture growth medium and recentrifuged. A concentrate of cells for implantation was finally prepared by resuspending the cells in a minimal amount of growth medium.

Tissue disaggregation using proteolytic enzymes. Liver tissue was cut into small fragments and transferred into a trypsinisation flask. The action of 2 enzymes on liver tissue was examined, a protease prepared from *Streptomyces griseus* ("Pronase"), and trypsin.

"Pronase" grade B, containing 45,000 proteolytic units per g. was prepared by stirring for 2 hr the powdered form of the protease in a modified Hanks' balanced salt solution free from calcium and magnesium ions on a hot plate maintained at about 30°. The solution was filtered through a grade 5 sintered glass filter and stored at −20° until required. Liver fragments were magnetically stirred in a flask at room temperature for 20 min. The cell suspension was passed through 2 layers of gauze into a tube containing calf serum to yield a 20 per cent final concentration of serum in the cell suspension in order to arrest the action of the enzyme. The suspension was centrifuged at 500 rpm for 5 min. at 20°. The deposit was washed in the growth medium then centrifuged twice before implantation. It appeared that exposure of the tissue to "Pronase" for 20 min. somewhat damaged the hepatic cells and a modified method was subsequently employed. Small pieces of liver tissue were aspirated up and down with a wide bore pipette in 0·1 per cent solution of "Pronase" for 3–4 min until a fine suspension of cells was obtained. Centrifugation and washing as outlined above was repeated, but it was soon noted that the cells tended to aggregate into large clumps after washing in the growth medium, and it was necessary to resuspend the cells in Alsever's solution before washing.

The second proteolytic enzyme used for cell dispersion was trypsin. Stock trypsin solution was diluted in modified Hanks' BSS free of magnesium and calcium ions at pH 7·6 to give a final concentration of 0·25 per cent trypsin. Small fragments of liver about 1 cu. mm. were added to the trypsin solution and magnetically stirred slowly at room temperature for 20 min. Rapid stirring resulted in digestion of the cells. A second and a third cycle of trypsinisation, using fresh trypsin solution, each lasting 20 min. were also attempted.

Calf serum giving a final concentration of 20 per cent was added to the cell suspension which was subsequently passed through gauze and centrifuged at 500 rpm at 4° for 5 min. The packed cells were washed in the growth medium, centrifuged once and resuspended in a minimal amount of the growth medium.

Tissue culture growth medium

Various growth media were investigated during the course of these experiments. Survival of liver cells was found to be most consistent in growth medium based on the following formula: 90 per cent Eagle's minimum essential medium, 10 per cent foetal calf serum, 0·15 per cent sodium bicarbonate solution, 100 units per ml. penicillin, 100 μg. per ml. streptomycin.

The covered tissue culture plates were placed in a sealed desiccator with about 2 per cent added CO_2, and incubated at 35°. The growth medium was changed 3 times weekly.

Aflatoxin

Purified aflatoxin B_1 (500 μg.), which was kept in the dark at 4° until required, was dissolved in 0·04 ml. of dimethyl formamide and diluted in the growth medium to give a final concentration of 10 p.p. million (10 μg./ml.) in the tissue culture growth medium. In other experiments the final concentration of aflatoxin B_1 was 5 p.p. million (5 μg./ml.) and 1 p.p. million (1 μg./ml.). The diluent, dimethyl formamide, was used in control preparations in parallel with preparations inoculated with aflatoxin while other implants were cultured in the growth medium alone.

Staining methods and microscopy

Implants were stained with 1/1000 (w/v) solution of acridine orange in acetate veronal buffer pH 4·2 for study by fluorescence microscopy. Single disc implants were removed for examination at intervals from the tissue culture plates and floated on the stain with the tissue downwards for 20 min. No fixation was employed. The preparations were washed for 10 min. in acetate veronal buffer pH 4·2 with three changes of buffer, and then mounted in 5 per cent (v/v) glycerol in water. A Reichert fluorescence microscope was used for examining preparations stained with acridine orange. The source of ultraviolet light was an Osram HBO 200 mercury vapour lamp. The following filters were used, an E3 (BG 12/6 mm.) exciter filter against the mercury lamp and an Sp 3 (GG 9/1 mm. + OG 1/1·5 mm.) barrier filter in the monocular eyepiece. The implants were examined using a dark ground condenser, a × 63 dry objective and a × 8 ocular.

Standard histochemical techniques were employed for examining the liver preparations for fat (Sudan III and IV method for neutral fats after Kay and Whitehead, and the Oil Red O method after Lillie); for glycogen Best's carmine stain was used and the Periodic Acid-Schiff technique for glycoproteins. Perl's method was used for staining for ferric iron. Adenosine triphosphatase activity was localized by the method of Wachstein and Meisel.

RESULTS

Tissue culture

Mechanical disaggregation of the tissue on the whole gave poor results since the clumps of liver cells so obtained were too large and implantation proved to be difficult without causing considerable trauma to the cells. The immediate survival of cells by this technique was roughly estimated at only 30–40 per cent of the total cell population implanted. A poor survival rate beyond 72 hr. was the result of the use of "Pronase" by either method, although somewhat better results were obtained when the enzyme was allowed to act on the cells for only a few minutes. Small size cell aggregates were obtained but nevertheless these proved to be too thick for satisfactory implantation.

Trypsinisation gave the best results and the cells could be easily implanted to give an almost uniform monolayer of viable cells. Preparations obtained from cells of the second and third cycles of trypsinisation appeared at least as good as implants of cells from the first fraction. Examination of the tissue immediately after implantation (Fig. 1) and some hours later (Fig. 2) showed complete recovery of the cells from the trauma of the procedure.

The pressure required to reduce tissue fragments to a monolayer is critical particularly for soft tissues such as liver. Too great a pressure will invariably result in death of the cells. We employed a glass weight of 757 g. trimmed to the size of the Perspex plate so that the pressure exerted was 4·8 g./cm^2. The weight was applied for 40 sec. before clotting of the plasma occurred. However, with a little practice the effect of manual pressure applied can be judged directly by visual observation through the clear Perspex without recourse to actual pressure weights.

The sex of the embryo from which the liver was derived appeared to be immaterial. The age of the embryo, 8–22 weeks, again did not seem to be of importance in the short term study of the survival of the hepatic cells. The serum used in the growth medium, however, proved to be critical. Of the 4 samples of calf serum employed in the growth medium, whether or not inactivated at 56° for 30 min. before use, only one sample was found to support the survival of hepatic cells when used at a concentration of 20 per cent. Human serum at a concentration of either 10 or 20 per cent in Eagle's minimum essential medium did not prove satisfactory. Consistent and reproducible results, however, were obtained with 10 per cent foetal calf serum.

Survival of the implanted tissue was assessed by examination of preparations by fluorescence microscopy and by study of the cells under phase-contrast illumination. It is estimated that approximately 90 per cent of the hepatic cells survived in our preparations for periods varying from 10–16 days. When hepatic cell degeneration and death occurred this tended, on the whole, to be rapid and fairly uniform throughout the implanted tissue.

Complete recovery of the cells from the trauma of implantation occurred within several hours. Although there was evidence of some cellular trauma (Fig. 1) disruption of the cytoplasm or of the nuclear membrane did not occur. After overnight incubation the implant assumed the normal appearance of hepatic tissue, with most of the cells arranged in a monolayer. After 4–8 days many hepatic cells acquire cytoplasmic vacuoles and green fluorescing cytoplasmic granules (Figs. 3 and 4). On further maturation the cytoplasm becomes loaded with these granules. At the same time the hepatic cells gradually increase in overall dimensions, and many attain considerable size after 10–12 days. Histochemical staining has shown these granules to consist of neutral fat. The hepatic cells also contained glycogen.

Active division of the hepatic cells does occur (Figs. 4 and 5) but frequent mitotic figures were not observed in the preparations. An expected problem with liver in tissue culture, which was encountered, is that associated with the pluripotential nature of the cells, particularly of the supporting connective tissue cells. Fibroblast-like cells begin to dominate the picture after about 10 days in culture, and on occasion sheets of fibroblasts tend to overgrow the hepatic tissue. Bile duct canaliculi were identified in our preparations by ATP-ase activity using Wachstein and Meisel's method. Macrophages and monocytes though scarce in the first few days of culture rapidly increase in number after about 9 days. The cytoplasm of these cells was found to contain considerable amounts of haemosiderin granules.

The cytotoxicity of aflatoxin B_1

The earliest microscopic changes were observed after 3–5 hr exposure to aflatoxin in a concentration of 10 p.p. million. These consisted of a change in the normal granular appearance of RNA in the cytoplasm and loss of definition of the nucleoli. Marked cytopathic changes were observed after 16 hr exposure. The overall dimensions of the hepatic cells were reduced and the cells rounded up. There was complete loss of orange (RNA) fluorescence from the cytoplasm, the cytoplasm was opaque and now fluoresced deep emerald green. The nucleoli could no longer be identified and there was apparent loss of the chromatin material of the nucleus which normally stains light green but now became a deep opaque orange in colour (Fig. 6). These changes were very striking and consisted of a complete reversal of the normal appearance of the hepatic cells when examined by fluorescence microscopy. The cytopathic changes became progressively more marked with time to ultimate death of the cells and ghost-appearance after 40–44 hr. At a concentration of 5 p.p. million of aflatoxin B_1 no obvious changes in the cultures were apparent until 20–24 hr. exposure when the changes described above rapidly took place (Table). The characteristic effect of the toxin, at a concentration of 1 p.p. million, was evident in about 50 per cent of the cells in preparations examined after exposure to the toxin for 24 hr. Control preparations

EXPLANATION OF PLATE

Fig. 1.—The appearance of the hepatic tissue immediately after implantation. × 400.

Fig. 2.—A monolayer of hepatic cells 48 hr after implantation. × 160.

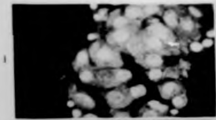
Fig. 3.—Fibroblasts contrast sharply with the hepatic cells and are easily identified. Note the early appearance of granules and vacuoles in the hepatic cells and the increase in overall dimensions of the hepatic cells. × 400.

Fig. 4.—Mitosis in a parenchymal cell. Seventh day of culture. Vacuoles and green fluorescing granules are present throughout the tissue. × 400.

Fig. 5.—Sixteen days after implantation. A mitotic figure (poorly defined at bottom, right of centre) and surviving cells are present. × 400.

Fig. 6.—The characteristic cytotoxic effect of aflatoxin B_1. Note the greatly reduced dimensions of the hepatic cells and the complete reversal of normal fluorescence, i.e. the cytoplasm is deep green whereas the nucleus is orange. Compare with the control preparation (Fig. 2). × 400.

1



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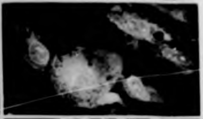

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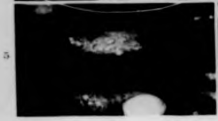

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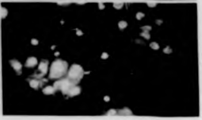

[illegible], [illegible] and Fulton.

with and without the diluent, dimethyl formamide, remained healthy and normal in appearance throughout the experiments (Fig. 2).

TABLE. *The Effect of Aflatoxin B_1 on Preparations of Human Liver Cells in Tissue Culture.*

	Concentration of aflatoxin					
	10 γ/2 ml/flask (5 μg/ml)		5 γ/2 ml/flask (2.5 μg/ml)			
Time intervals (h)	No. examined	No. with cytopathic changes	No. examined	No. with cytopathic changes	Diluent controls No. examined	Controls No. examined
3–5	8	5	6	6	3	7
16–24	5	3	14	14	12	13
25–48	10	10	5	9	16	16

Implantation and culture of liver tissue achieved by the technique we describe provides, firstly, a monolayer of cells without destruction of the normal architecture of the tissue. Secondly, it is not necessary to await active multiplication of the cells to form a tissue monolayer since the cells normally recover from the trauma involved in implantation within a few hours, and the preparation can then be used for experimental study. A third factor, and this applies particularly to short term experiments, is that the cells of the solid organ implanted have not undergone changes in morphology, differentiation or transformation. In fact although mitoses were not frequently observed in our preparations, this by itself is not a disadvantage supporting as it does our contention that differentiation or transformation has not occurred to any significant degree. A practical application of this technique was to study the influence of aflatoxin B_1 on human embryo liver cells.

There is strong suggestive evidence of the importance of naturally occurring hepatotoxins in the aetiology of human liver disease such as veno-occlusive disease, cirrhosis and primary carcinoma of the liver. For example, veno-occlusive disease of the liver as it occurs in the West Indies, Africa and the Middle East is believed to be due to the ingestion of "bush teas" and herbal remedies. Oettlé (1965) attempted to correlate the epidemiology of primary carcinoma of the liver which is exceptionally common in certain geographical areas of Africa and Asia with the possibility of consumption of food contaminated with the metabolic products of fungal origin. Oettlé drew attention to the high relative humidity of over 80 per cent which fungi require for growth and which indeed most of the regions concerned provide as well as rather primitive bulk food storage methods that would favour fungal contamination. Moreover, the significance of natural hepatotoxins in the more sophisticated communities should not be overlooked (Schoental, 1961). Cryptogenic cirrhosis still remains a problem in most countries, and furthermore the incidence of hepatoma seems to be increasing (Neame and Pillay, 1964). In experimental animals the aflatoxins have been shown to cause liver damage and indeed aflatoxin B_1 was found to be a potent carcinogenic agent. While it may be possible to translate experimental results from animals to man since there are many parallels between the experimental lesions with the pathological changes found in man, the direct effect of aflatoxin on human liver cells has not been previously observed. The cytotoxicity of aflatoxin B_1 for human liver cells is dramatic and it was found to be active at very low concentrations. The actual biochemical mechanism involved, however, has still to be elucidated and is under study at present.

SUMMARY

Monolayers of differentiated parenchymal human embryo liver cells were maintained in culture for periods varying from 10–16 days after implantation on polythene. Cell disaggregation by trypsinisation gave the best results. Survival of the liver cells was found to be most consistent in growth medium containing 10 per cent foetal calf serum. Preparations were examined at intervals by fluorescence microscopy after staining with 1:1000 acridine orange. Complete recovery of the cells after implantation occurred rapidly, and the normal appearance of hepatic tissue was assumed after overnight incubation. After four to eight days many granules of neutral fat accumulated in the cytoplasm of the hepatic cells.

When hepatic cell degeneration and death occurred this tended to be rapid and fairly uniform throughout the implanted tissue. Fibroblasts increase in number after about 10 days in culture and on occasions sheets of fibroblasts overgrow the hepatic tissue.

The effect of purified aflatoxin B_1 on the liver cells was investigated. Marked changes were observed after 16 hr. exposure of the cells to 10 p.p. million (10 μg./ml.) of aflatoxin B_1. The overall dimensions of the hepatic cells were reduced. There was complete loss of orange (RNA) fluorescence from the cytoplasm, and the cytoplasm became opaque and fluoresced deep green. The nucleus also showed marked changes and death of the cells followed.

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